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Protein Phosphatases 2C of *Arabidopsis thaliana* in Stress Signaling

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Für meine Familie …

Contents

List of Figures and Tables

Figures

Tables

Abbreviations

2.4-D	2,4-Dichlorophenoxyacetic acid
ABA	Abscisic Acid
ABF	ABRE binding factor
ABI1/2	Abscisic Acid Insensitive 1/2
ABRE	ABA Response Element
ACC	1-Aminocyclopropane-1-carboxylic acid
AD	Activation Domain
AHG1	ABA-hypersensitive Germination 1
AID	Autoinhibitory Domain
AKT1	Arabidopsis Potassium Transporter 1
Amp	Ampicillin
BD	DNA Binding Domain
Bet v 1	Major birch pollen allergen of *Betula verrucosa* 1
CA	constitutively active
CaMV	Cauliflower Mosaic Virus
CBL	Calcineurin B like
CID	CBL-interaction domain
CIPK	CBL-interacting protein kinase
CLV1	CLAVATA1
CPK	Ca^{2+}-dependent protein kinase
CPK23t	truncated CPK23
CSBP	Cytokinin Specific Binding Protein
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
emp	empty vector
EtBr	Ethidium Bromide
Eth	Ethylene
EtOH	Ethanol
GA3	Gibberellic Acid 3
GAL4	Galactose Metabolism 4
GUS	β-Glucoronidase
H	Histidine
HAB1/2	Homology to ABI 1/2
HAI1/2/3	Highly ABA-induced PP2C 1/2/3
HM	Hormone Mixture
HR	Hypersensitive Response
IAA	Indole-Acetic-Acid
IC_{50}	50 %-inhibitory concentration
IPTG	Isopropyl-β-D-thiogalactopyranosid
JA	Jasmonic Acid
Kan	Kanamycin
L	Leucine
LIG	Ligand
LUC	Luciferase
M	Methionine

MAPK	Mitogen Activated Protein Kinase
MCS	Multiple Cloning Site
MeJA	Methyl-Jasmonic Acid
MLP	Major Latex Protein
MUP	Methylumbelliferyl Phosphate
O/N	over night
OD	Optical Density
ONPG	o-Nitrophenyl-β-D-galactopyranosid
OST1	Open Stomata 1
PAMP	Pathogen Associated Molecular Patterns
PAPP2C	Phytochrome Associated PP2C
PDB	Protein Data Bank
PE	Plant Cell Ectract
PEG	Polyethylene Glycol
PIA1	PP2C induced by AvrRpm1
PipA	Pipecolic Acid
PLL	POL-like
POL	Poltergeist
PP2C	Protein Phosphatase Type 2C
PR1	Pathogenesis-Related 1
PYR1	Pyrabactin Resistance 1
RCAR	Regulatory Components of ABA Receptor
RD29B	Responsive to Desiccation 29B
RFU	Relative Fluorescence Unit
Rif	Rifampicin
RLU	Relative Luminescence Unit
ROS	Reactive Oxygen Species
SA	Salicylic Acid
SD	Synthetic Dropout
SDM	Site Directed Mutagenesis
SDS	Sodium Dodecyl Sulfate
SLAC1	Slow Anion Channel 1
SnRK2	Sucrose non fermenting 1-Related Kinase 2
START	Star-related lipid transfer domain
TAIR	The Arabidopsis Information Ressource
Tet	Tetracyclin
TF	Transcription Factor
TMV	Tobacco Mosaic Virus
UBQ10	Ubiquitin 10
W	Tryptophane
WIN2	HopW1-1 interacting protein 2
wt	wild type
Y2/3H	Yeast-two/three-hybrid
YLGA	Y2H Liquid culture Growth Assay
β-Gal	β-Galactosidase
βME	β-Mercapto-Ethanol

Abstract

Protein phosphatases type 2C (PP2Cs) are major regulators of diverse signaling pathways in all organisms. Particularly in higher plants, this protein family has expanded in number and diversified in function. Studies on the model plant *Arabidopsis thaliana* contributed to the understanding of vital signaling cascades controlled by PP2Cs.

In abiotic stress response, governed by the phytohormone abscisic acid (ABA), PP2Cs of subgroup A are major negative regulators. The identification of the core regulatory module was pivotal for the understanding of the molecular machinery driving plant stress responses. In this work, the combinatorial assembly of all possible ABA co-receptor complexes between 9 clade A PP2Cs and 14 Regulatory Components of ABA Receptor (RCARs) was tested in yeast. The results propose ABA-dependent or ABA-independent complex formation for certain combinations. In transient protoplast expression studies, the effects of PP2Cs, RCARs and a combination of both were studied *in vivo*. These results confirmed the heterologous interaction analyses, and outlined a subset of conceivable interactions for the control of ABA signaling *in planta*. The opposing function of PP2Cs and RCARs is also reflected at the transcriptional level. PP2Cs are rapidly and strongly induced by ABA-mediated stress signaling. RCAR transcripts are significantly down-regulated under the corresponding conditions.

In ABA-related signaling, PP2Cs directly control a number of downstream targets such as protein kinases and ion channels. Among these targets, there are the CBL-interacting protein kinases (CIPKs). Analyses of the complement of possible interactions between PP2Cs and CIPKs in yeast confirmed preferential and specific coupling. The expression of constitutively active CIPK (CA) versions in protoplasts revealed an effect on ABA-dependent signal transduction for at least three of the CIPKs. Also, some members of the Ca^{2+}-dependent protein kinase family (CPKs) are directly interfering with PP2Cs from clade A. Here, CPK23 was identified as a PP2C-interacting CPK, which interferes with PP2C binding to RCARs and SnRK2s, in a kinase activity-dependent manner.

To test a possible conservation of a PP2C-RCAR-like signaling relay in other signaling pathways, 23 PP2Cs from different subgroups and 6 RCAR-related Major Latex Proteins (MLPs) were analyzed for interaction in a heterologous system. This approach led to the identification of a WIN2-MLP34 complex, probably involved in biotic stress signaling. As a potential ligand for this receptor complex is not known, the function of MLPs and PP2Cs was tested in salicylic acid (SA)-dependent signaling in protoplasts. At least five of the tested MLPs caused significant alterations in SA response, but detailed analyses on the function of these proteins in defense signaling have to be performed.

Zusammenfassung

Protein Phosphatasen des Typs 2C (PP2Cs) kontrollieren Stress-Signalwege in verschiedensten Organismen. Speziell in den höheren Pflanzen hat sich diese Proteinfamilie stark vergrößert und diversifiziert. Untersuchungen an der Modellpflanze *Arabidopsis thaliana* haben beträchtlich zum Verständnis von PP2C-gesteuerten Signalwegen beigetragen.

Die Reaktion auf abiotischen Stress wird vor allem durch das Pflanzenhormon Abszissinsäure (ABA) gesteuert. PP2Cs der Gruppe A fungieren hierbei als maßgebliche negative Regulatoren. Die Entdeckung des zentralen Signalmoduls ermöglichte die Aufklärung des molekularen Mechanismus der Stress-Antwort. Diese Arbeit untersucht alle möglichen Co-Rezeptor-Kombination aus 9 PP2Cs der Gruppe A und den 14 regulatorischen RCAR Proteinen in Hefe. Die identifizierten Interaktionen entstehen zudem teils nur in Anwesenheit des Liganden ABA. Die Funktion der diversen Komponenten wurde mittels transienter Expression in Protoplasten untersucht. Diese Ergebnisse stimmen mit den Hefe-Interaktionsdaten überein und verweisen auf die mögliche Funktion einzelner Interaktionen im ABA-Signalweg *in planta*. Außerdem zeigt sich die entgegengesetzte Regulation von PP2Cs und RCARs auf transkriptioneller Ebene. Bei ABA-vermitteltem Stress werden PP2Cs rasch hoch-reguliert, während RCARs deutlich herunter-reguliert sind.

In der ABA-gesteuerten Signalkaskade kontrollieren PP2Cs eine Reihe von Zielproteinen, wie Proteinkinasen oder Ionenkanäle. Darunter finden sich auch CBL-Interagierende Protein Kinasen (CIPKs). In Hefe zeigen sich spezifische Interaktionen zwischen PP2Cs und CIPKs. Zudem zeigen mindestens drei CIPKs einen deutlichen Effekt auf die ABA-abhängige Signaltransduktion in Protoplasten. Des Weiteren konnte eine direkte Wechselwirkung der PP2Cs mit Ca^{2+}-abhängigen Proteinkinasen (CPKs) nachgewiesen werden. CPK23 interagiert mit PP2Cs der Gruppe A und moduliert deren Wechselwirkung mit RCARs und anderen PP2C-Substratproteinen.

Um zu testen ob ein PP2C-RCAR-ähnliches Signalrelais auch für andere Signalwege konserviert ist, wurden 23 PP2Cs aus unterschiedlichen Untergruppen auf ihre Interaktion mit RCAR-verwandten Major Latex Proteinen (MLPs) in Hefe getestet. Diese Untersuchungen führten schließlich zur Identifizierung eines PP2C-MLP-Komplexes, der möglicherweise in der Antwort auf biotischen Stress eine Rolle spielt. Da ein möglicher Ligand für diesen Komplex nicht bekannt ist, wurde die Funktion der diversen MLPs und PP2Cs im Salicylsäure (SA)-Signalweg in Protoplasten untersucht. Hier zeigte sich, dass zumindest fünf der getesteten MLPs die SA-abhängige Signaltransduktion beeinflussen. Detaillierte Untersuchungen dieser Effekte könnten zum Verständnis der molekularen Funktion der MLPs und PP2Cs in der SA-vermittelten Abwehrreaktion beitragen

1 INTRODUCTION

Plants are sessile organisms that have to cope with numerous environmental challenges. These challenges tremendously influence development, growth, physiology and overall fitness. The thale cress *Arabidopsis thaliana* (L.) HEYNH. has been intensively studied, regarding physiological and molecular responses to stimuli and stresses of various kinds. Besides its favorable features as a model plant, the genome of Arabidopsis is fully sequenced (Initiative, 2000; Somerville and Koornneef, 2002). Using the thale cress experimental system, a multitude of findings over the past decades has fostered the understanding of fundamental molecular biological processes in plants.

1.1 Stress

Abiotic impositions caused by climatically and physico-chemically unfavorable conditions, materially restrict yield in agricultural systems around the world (Al-Kaisi et al., 2013; Krishnamurthy et al., 2011; Lobell et al., 2009; van Ittersum et al., 2013). At the same time, plants are facing biological interference. Pathogens and diseases are hampering the growth and development of plants and are thereby reducing crop and food production (Chakraborty and Newton, 2011; Chang et al., 2013; Murray et al., 2013). Any unfavorable conditions or substances impeding the plant´s metabolism, growth or development, is to be regarded as stress (Lichtenthaler, 1996). An organism responds at a cellular level to such stresses trying to restitute and adapt. If the plant successfully reorganizes its metabolism, a resistance is achieved. When the intensity or duration of the stress is too high or long, respectively, it might result in permanent cellular damage or even death (Larcher, 1987). Particularly land plants, also known as embryophytes, have evolved a high degree of developmental and metabolic plasticity. They are able to rapidly react to environmental challenges. Therefore, the plant has to sense, integrate and transduce external biotic and abiotic signals in order to adjust its growth and development.

A common toolbox of components is involved in the integration of exo- and endogenous signals across divergent phyla. Variable conditions are sensed via receptor molecules and the activation of the latter triggers molecular and cellular responses in the plant cell. The required signaling is to a great extent mediated through the interplay of a variety of plant hormones and secondary messengers governing all these processes.

Phytohormones are bioactive, organic substances of low molecular weight. Some of these molecules, such as abscisic acid (ABA) and salicylic acid (SA), have been well-known for decades to be involved in

abiotic and biotic stress responses, respectively. For instance, ABA was identified as a substance important for fruit and leaf abscission (Addicott et al., 1968; Ohkuma et al., 1963). It accumulates in the plant cells upon stress or developmental stimuli and it is a crucial regulator of various traits such as germination, senescence or stomatal response to water shortage (Addicott and Lyon, 1969; Karssen et al., 1983; Zhang et al., 1987). On the other hand, biotic stress response functions mainly via an SA-dependent signaling cascade and it includes the generation of reactive oxygen species (ROS) as a first line of defense (Cameron et al., 1999; Lamb and Dixon, 1997). Aptly, the same secondary messengers (Ca^{2+}, ROS, etc.) are important in the reaction to varying and distinct environmental triggers (Bhattacharjee, 2005; Boudsocq and Sheen, 2012; Klusener et al., 2002; Kwak et al., 2006; Lecourieux et al., 2006). Throughout evolution a variety of mechanisms to achieve prompt activation and adequate control of cellular stress response pathways has evolved.

1.2 Protein dephosphorylation

On a molecular level, one way to activate response and adaption is the reversible phosphorylation of proteins. It is a fast and specific post-translational modification regulating protein function. This mechanism is conserved across the kingdoms. Through this modification, the cell has the possibility to rapidly control enzymatic function, transduce external and internal stimuli and alter physiological responses from the molecular up to the organismal level. The major players are the large family of protein kinases (PKs) and protein phosphatases (PPs). PKs transfer a phosphate group onto target residues, while PPs remove the latter. The amino acids usually targeted in the protein are serine, threonine or tyrosine.

1.2.1 Classification of protein phosphatases

Protein phosphatases functionally antagonize PKs and are categorized depending on their biochemical properties. PPs are not as specific as kinases concerning the recognition of target proteins (Ubersax and Ferrell, 2007). Usually phosphatases have a wider range of enzymatic substrates within the cell (Roy and Cyert, 2009). Most of the plant PPs belong to the family of serine/threonine (S/T) PPs. The other PPs are defined as protein tyrosine phosphatases (PTPs) or aspartic acid (D)-based phosphatases (Yang et al., 2010). S/T-PPs can further be classified depending on the requirement of divalent cations, sensitivity to peptide and molecular inhibitors and also based on their similarity in sequence and structure. PP1 and PP2A enzymes build the group of phosphoprotein phosphatases (PPPs). In animals, this group also comprises Ca^{2+}-dependent PP2Bs, but so far no member of this subfamily has been identified in plants. Sensitivity to inhibitors like

okadaic acid and microcystin is characteristic for PP1 and PP2A proteins, whereas PP2B and PP2C are insensitive to those substances. Similar to PP1s, PP2As are heteromeric enzymes composed of a number of different regulatory and catalytic subunits. The largest family of S/T-PPs in plants is the metal-dependent PPs (PPMs), also known as protein phosphatases 2C (PP2Cs). These monomeric enzymes require Mg^{2+} or Mn^{2+} cations for activity. They are also insensitive to known PP1 inhibitors. Structurally PP2Cs are related to the PPPs, but there is no sequence homology (Cohen, 1989).

Compared to humans, the quantity of plant PPs in the different subfamilies is diverging (Table 1). The numbers of genes encoding catalytic subunits of PP1 and PP2A are comparable. In the human genome, three PP1 and two PP2A catalytic isoforms are encoded, in Arabidopsis there are nine and five genes, respectively (Kerk et al., 2008; Moorhead et al., 2007). This relatively small number of catalytic subunits is complemented by a great number of regulatory and structural subunits (>100 in humans and 20 - 30 in Arabidopsis and rice). The combinatorial assembly of these subunits gives rise to a plethora of possible holoenzymes, each of which having a specific function (Eichhorn et al., 2009; Farkas et al., 2007).

Table 1: Classification and summary of PP genes in rice, *Arabidopsis thaliana* (Ath) and humans. Based on Kerk et al. (2008); Moorhead et al. (2009); Sacco et al. (2012); Yang et al. (2010).

PP	Class		# rice	# Ath	# human
D-based	FCP-like / CPL		21	25	6
	HAD family		3	4	11
PTP	class I PTP		1	1	41
	class I DSP		22	18	63
	class II PTP (CDC25)		0	0	3
	class III PTP (LMW)		2	2	1
	class IV PTP		4	5	4
S/T-PP	PPP family	PP1 [ab]	5	9	3
		PP2A [ab]	21	5	2
		PP2B [cd]	0	0	3
		Others	10	15	5
	PPM / PP2C [de]		**90**	**80**	**17**

a) senstitive to inhibitors, b) not requiring cations for activity, c) Ca^{2+}-dependent acitivity, d) insensitive to inhibitors, e) Mg^{2+}/Mn^{2+}-dependent activity

Strikingly, the number of mammalian PP2Cs is small, with 17 members compared to the 80 and 90 genes identified in Arabidopsis and rice, respectively (Singh et al., 2010; Xue et al., 2008). Particularly in plants, the PP2Cs have emerged as major players in stress signaling and response pathways (Schweighofer et al., 2004).

1.3 PP2Cs in stress signal transduction

The fundamental function of plant PP2Cs centrally controlling major stress response pathways in higher plants is based on their evolutionary radiation and specification. Compared to prokaryotes and also other eukaryotes, plants have evolved a comparably large number of PP2Cs in their genomes. This particular family of S/T phosphatases has expanded significantly in higher plants,

possibly reflecting the diverse range of environmental cues effecting plant life. Surprisingly, the central function of PP2Cs as important regulators of stress signaling seems to be conserved across kingdoms. Archaea, bacteria, unicellular eukaryotes and higher organisms such as metazoans and plants, all share a common set of modules in PP2C-related signaling cascades (Fig. 1).

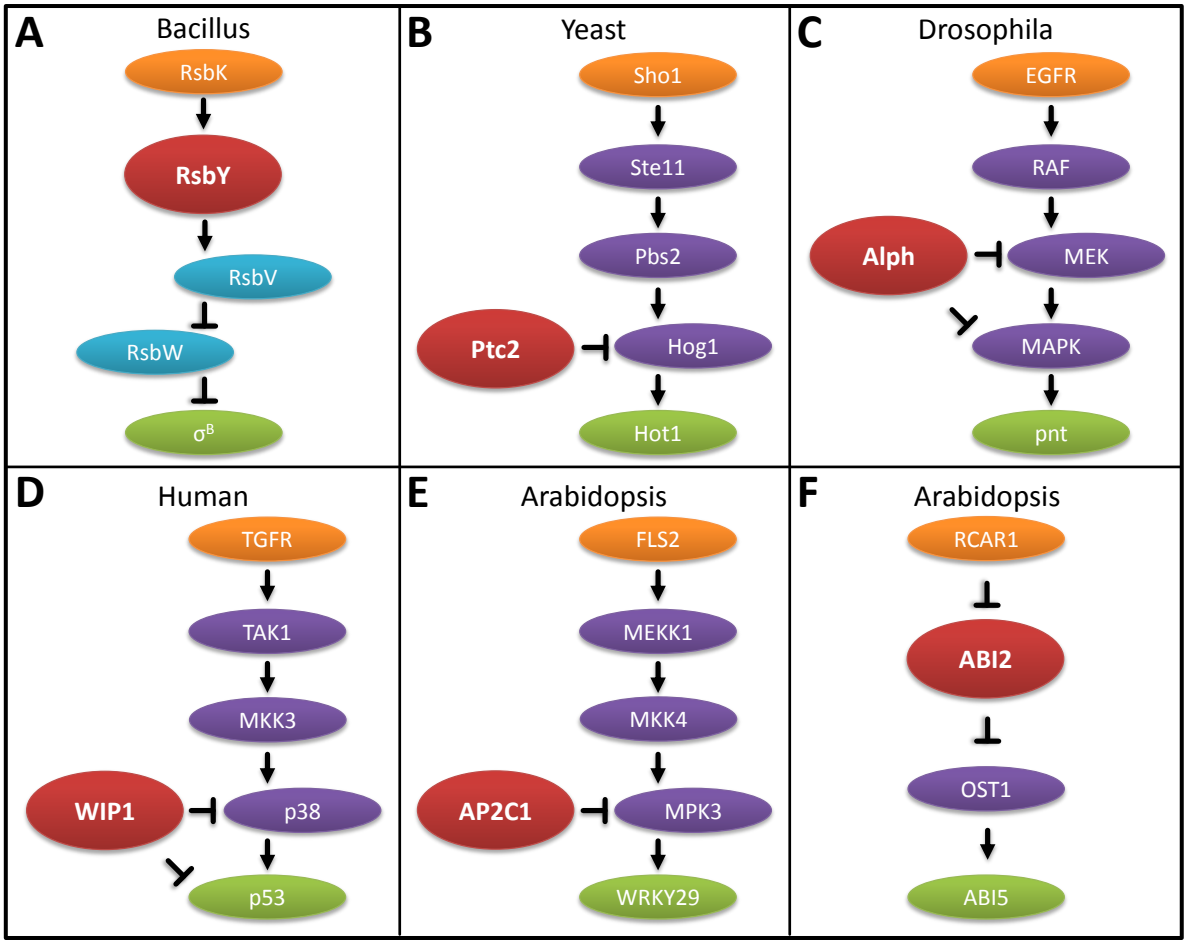


Fig. 1: Modular regulation of PP2C-controlled stress response pathways. Sensory proteins (colored in orange) perceiving the stress stimulus invoke cellular signal transduction. Under unstressed conditions PP2Cs (red) oppose and inhibit downstream kinases (purple). Upon elicitation, the PP2C constraint is removed and subsequently transcriptional regulators (green) are activated and effect differential expression of target genes. (A-F) Exemplary signaling pathways controlled by PP2Cs in various organisms are schematically presented (for details see text). Modified from Fuchs et al. (2012)

In the bacterial energy stress pathway, a PP2C positively regulates the malnutrition response. The σ^B transcription factor (TF) is eventually activated after PP2C-dependent inactivation of the anti-σ-factor (Brody et al., 2009; Kim et al., 2004b). In all the other stress response cascades depicted, the PP2C functions as a negative regulator. In eukaryotes, the protein phosphatase normally inhibits kinases of various kinds and thereby blocks downstream signaling. Upon stress perception, the PP2C is removed from the system to release the inhibition of downstream kinases. Phosphorylation of the target proteins ultimately leads to activation of stress response, e.g. transcriptional reprogramming. The

minimal set of common components comprises of the stress sensor, protein kinases mutually controlled by PP2Cs and a transcriptional regulator.

In Fig. 1 (A) the scheme of the energy stress response pathway of *Bacillus cereus* is depicted. The sensory protein RsbK activates the PP2C RsbY. Active RsbY dephosphorylates anti-anti-σ-factor RsbV and releases σ^B from RsbW-mediated inhibition (Chen et al., 2012). (B) In the yeast *Saccharomyces cerevisiae,* osmotic stress triggers a typical Mitogen Activated Protein Kinase (MAPK) signaling cascade. Stimulation of MAPKs results from activation by upstream MAPK kinases (MAPKKs) and MAPKK kinases (MAPKKKs). In this case, hyperosmolarity induces the High Osmolarity Glycerol 1 (Hog1) MAPK cascade. The Sensor for High Osmolarity 1 (Sho1) activates MAPKKK Sterile 11 (Ste11). The signal is passed on via phosphorylation on Polymyxin B Sensitivity 2 (Pbs2) MAPKK and Hog1 MAPK to the High Osmolarity-induced Transcription 1 (Hot1) transcription factor. The PP2C family member Phosphatase Two C 2 (Ptc2) blocks signal transduction at the Hog1 MAPK level (Mapes and Ota, 2004; Young et al., 2002). (C) A MAPK cascade steering development in the fruit fly *Drosophila melanogaster* is controlled by Alphabet (Alph) PP2C. The PP2C counteracts MEK (MAPKK) and MAPK function, which are launched by a Epidermal Growth Factor Receptor (EGFR)-dependent Recombinase Activating Factor (RAF) MAPKKK activation. If Alph is active, the Pointed (pnt) transcription factor remains inactive (Baril and Therrien, 2006; Gaengel and Mlodzik, 2003). (D) In human cells genotoxic agents and irradiation lead to the activation of the p53 transcriptional regulator, controlling proliferation and apoptosis. Wild-type p53-Induced Phosphatase 1 (WIP1) PP2C interferes with p38 MAPK and p53 TF function directly. Under stress conditions, the signal is perceived by the Transforming Growth Factor Receptor (TGFR) and transduced to MAPKK 3 (MKK3) via TGFβ Activated Kinase 1 (TAK1) MAPKKK activation (Cuadrado and Nebreda, 2010; Goloudina et al., 2012; Lu et al., 2005; Xia et al., 2011). (E) In *A. thaliana* the Flagellin Sensitive 2 (FLS2) receptor like kinase (RLK) senses biotic stress and elicits transcriptional response via the MEKK1 (MAPKKK), MKK4 (MAPKK), MPK3 (MAPK) cascade. Eventually, transcription factors such as WRKY29 elaborate altered gene expression (Asai et al., 2002; Chinchilla et al., 2007; Qiu et al., 2008; Rodriguez et al., 2010). (F) Drought stress leads to the accumulation of ABA in the plant cell. ABA is bound by the RCAR1 sensor and establishes a heterotrimeric complex between the ABI2 PP2C, the receptor RCAR and the phytohormone. In turn, this hormone binding releases downstream kinases such as Open Stomata 1 (OST1) from PP2C inhibition and ABA-responsive gene expression is driven by ABI5 action. (Klingler et al., 2010; Raghavendra et al., 2010; Umezawa et al., 2010).

1.3.1 Evolution of the PP2C family

Type 2C protein phosphatases are evolutionary conserved across kingdoms. PP2C genes are present in archaea, bacteria and eukaryotes such as yeast, rice and humans. The major function of this protein family in the regulation of stress response is conserved in different species and different pathways (compare Fig. 1). In the course of evolution, PP2C genes have largely diversified. The total number of PP2C genes ranges from one or very few copies in the genomes of archaea (e.g. *Thermococcus sp.*) (Dahche et al., 2009) or of bacteria (e.g. *Bacillus sp.*) (Shakir et al., 2010) up to more than 100 proteins in cultivated plant species such as potato (*Solanum tuberosum*) or maize (*Zea mays*) (Fuchs et al., 2012). In yeast and metazoans, the diversification does not come up to the multitude of PP2Cs found in plants. Thus, the radiation of plants and the evolution of more complex organisms that have to cope with a variety of ever changing environmental conditions, is illustrated by the boost of total PP2C numbers per organism (Fig. 2). While the genome sizes of primal genera like *Clamydomonas*, *Physcomitrella* and *Seleginella* (0,12 Gbp - 0,14 Gbp) (Little et al., 2007; Rensing et al., 2008) are similar to those of plants like Arabidopsis and rice (0,14 Gbp - 0,46 Gbp) (Initiative, 2000; Yu et al., 2002), the number of PP2Cs per genome increased markedly. Generally, with the increasing complexity of the organisms and multifaceted environmental challenges, the number of PP2Cs has mounted throughout evolution. Particularly in green plants, the broad variety of PP2C proteins reflects physiological and morphological plasticity. For instance, the number of clade A PP2Cs, known to be important in ABA-dependent stress signaling, increased from early land plants to cultivated species. In *Physcomitrella patens* and *Selaginella moellendorffii* only a few clade A PP2Cs regulate drought response. In *Populus trichocarpa* and *Z. mays* the number of clade A PP2Cs has multiplied (Fuchs et al., 2012).

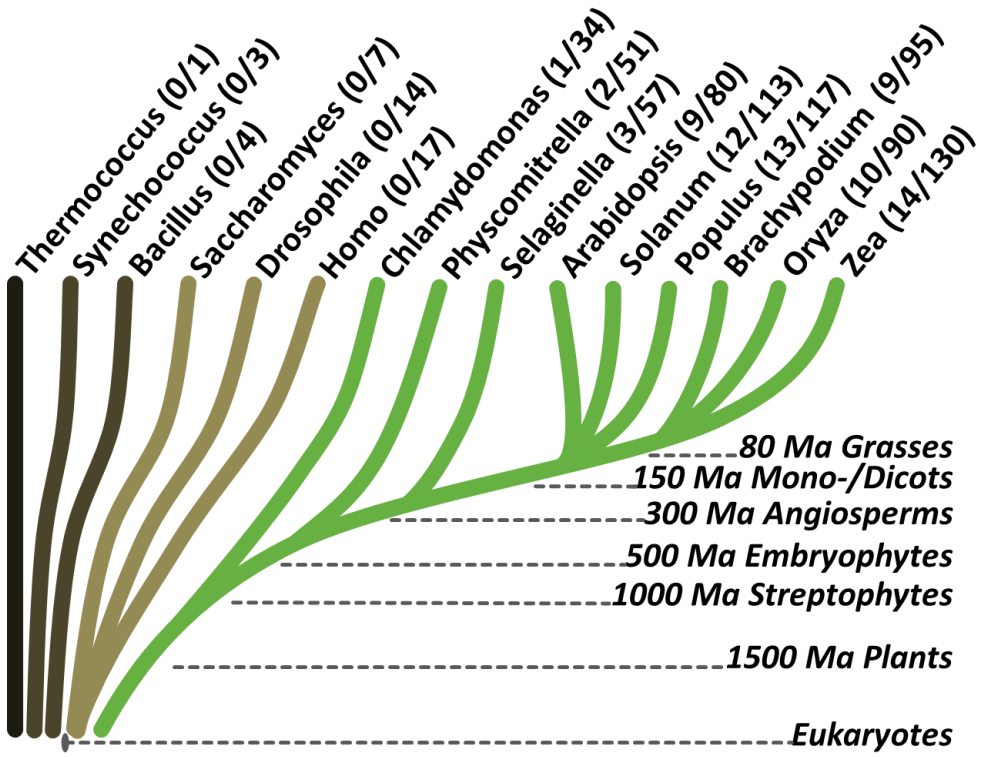


Fig. 2: Schematic evolutionary tree representing PP2C radiation. The estimated evolutionary timescale of plant diversification is indicated by dashed lines and given in million years [Ma]. The organisms representing different kingdoms (colored) in the tree are: *Thermococcus sp.*, Archaea (black); the cyanobacterium *Synechococcus sp.* and the eubacterium *Bacillus subtilis*, Bacteria (brown); the heterotrophs such as unicellular *S. cerevisiae* and the metazoans *D. melanogaster* and *Homo sapiens*; non-plant eukaryotes (sand); and the alga *Chlamydomonas reinhardtii*, the moss *P. patens*, the fern-like *S. moellendorffii*, the dicots *A. thaliana*, *S. tuberosum*, *P. trichocarpa*, and the monocots *Brachypodium distachyon*, *Oryza sativa* and *Z. mays*, plants (green). The numbers in parenthesis represent the number of clade A PP2Cs in the corresponding species. Modified from Fuchs et al. (2012).

1.3.2 The PP2C family in Arabidopsis thaliana

Fig. 3 shows a phylogenetic tree of the 80 protein phosphatases 2C annotated in the Arabidopsis genome (Xue et al., 2008; Yang et al., 2010). The family subdivides into 12 major clades, which are labeled A-L.

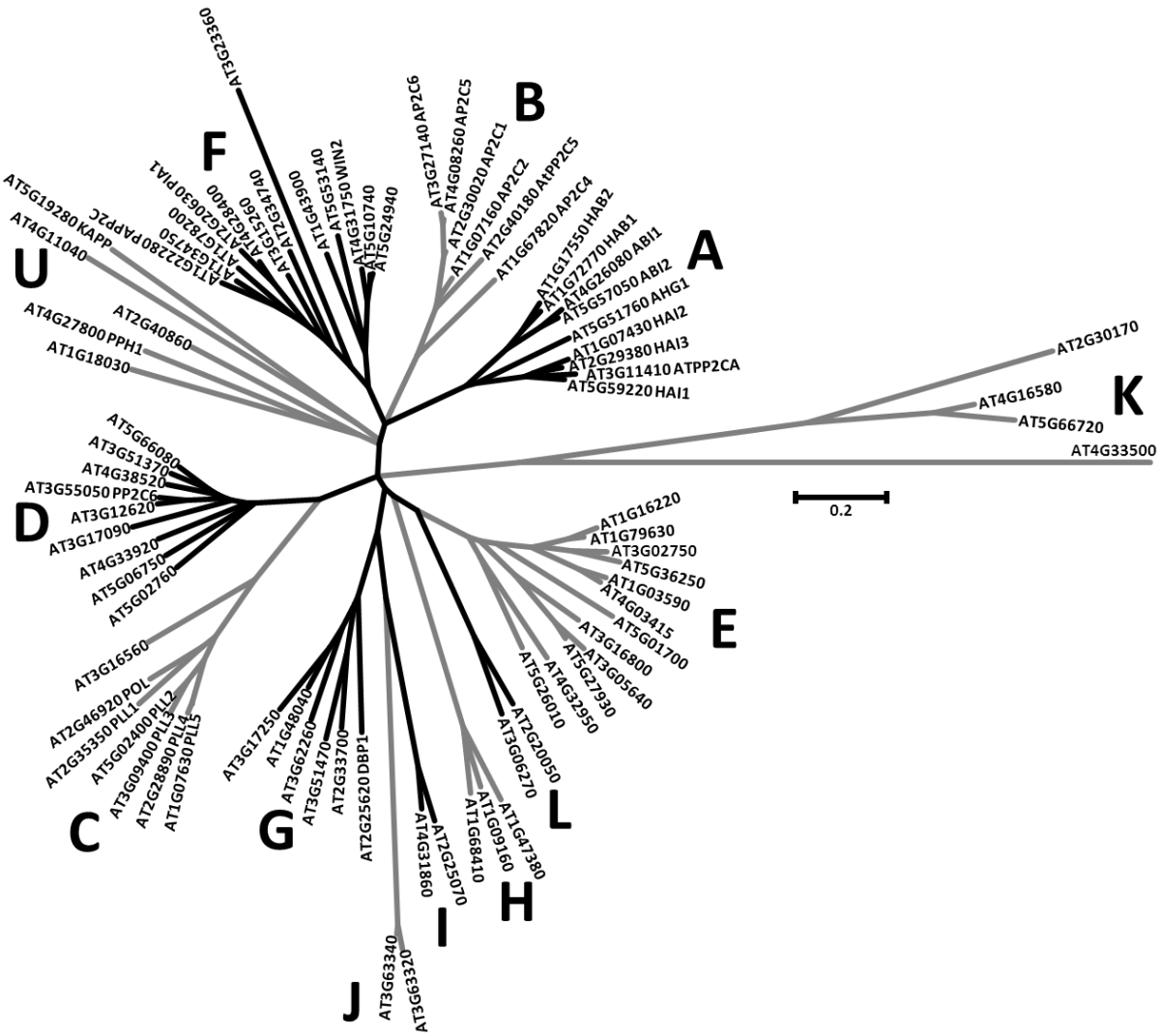


Fig. 3: Phylogenetic tree of the *A. thaliana* PP2C family encompassing 80 proteins. This unrooted Neighbour-Joining consensus tree was calculated using MEGA5 software (Tamura et al., 2011). The subfamilies (A-L), including the members originally termed "unclustered" (U), are labeled according to the clades defined by Schweighofer et al. (2004). Single PP2Cs are enumerated and provided with gene names, where applicable. Branch lengths indicate evolutionary distances corrected by Poisson transformation and are given in units of amino acid substitutions per site. Modified from Fuchs et al. (2012).

In Arabidopsis, several pathways entangled with the action of a PP2C have been identified. Surprisingly, the phylogenetic clustering (see Fig. 3) commonly reflects functional correlation amongst distinct subgroups. In the following, a few PP2C subfamilies and their involvement in well-known signaling cascades are presented, namely the groups B, C, F and A.

Clade B

The phosphatases of clade B are centrally involved in the control of MAPK cascades (see also Fig. 1). The six genes are named AP2C1 to AP2C6 and are homologous to the originally characterized

Medicago sativa PP2C-type phosphatase MP2C (Meskiene et al., 2003; Meskiene et al., 1998). The inactivation of MAPK-dependent signaling by the AP2Cs is achieved by dephosphorylation of a conserved pT in the kinase activation loop. AP2C1 controls MPK4- and MPK6-dependent signaling in wounding response and plant innate immunity (Schweighofer et al., 2007). AP2C3 plays a role in the development of stomatal lineage cells, through interference with MPK3 and MPK6 function. The deregulation of MPK signaling not only effects stomatal development and patterning (Umbrasaite et al., 2010; Wang et al., 2007), but also alters stress responsive gene expression (Brock et al., 2010).

Clade C

The POLTERGEIST (POL) and POLTERGEIST-like (PLL) PP2Cs govern the maintenance of stem-cell populations in meristamatic and embryonal tissues (Song and Clark, 2005). The members of this family show membrane association and they are activated by phospholipid binding (Gagne and Clark, 2010). The PP2Cs promote meristem development and sustainment. POL and PLL1 are part of the CLAVATA1 (CLV1) signaling cascade supporting WUSCHEL stem cell factor function (Song et al., 2006). Additionally, these two phosphatases are essential for root and shoot stem cell specification (Song et al., 2008). Mutants deficient in PLL4 or PLL5 PP2Cs exhibit strong phenotypes related to leaf development. The molecular function of other PLLs remains unknown.

Clade F

The proteins of this subfamily are defense-related PP2Cs that function in biotic stress response. The recognition of Pathogen Associated Molecular Patterns (PAMPs) by the plant, deploys a complex system of defense mechanisms (Asai et al., 2002; Boller and He, 2009; Zipfel et al., 2004). While the pathogen is trying to overcome host defense, the plant launches first local and consequently systemic responses to achieve resistance (Dangl and Jones, 2001; Suzuki et al., 2004). SA plays a major role in establishing systemic acquired resistance (SAR) (An and Mou, 2011; Cameron et al., 1999; Shah, 2003; Vlot et al., 2009). Besides pathogen-induced SAR signaling, general defense is also importantly mediated by other phytohormones like jasmonic acid (JA), ethylene (ET) and ABA (Adie et al., 2007; Anderson et al., 2004; Ludwig et al., 2005; O'Donnell et al., 2003; Truman et al., 2007). Nevertheless, PP2Cs of clade F were identified in several studies to be connected to SA-dependent resistance to pathogens. For instance, the injection of the bacterial effector AvrRpm1 during *Pseudomonas syringae* infection leads to a specific accumulation of PP2C induced by AvrRpm1 (PIA1) (Widjaja et al., 2010). However, in the pia1 mutant, SA and JA levels are reduced compared to wt after infection. Additionally, defense related transcript levels are altered in pia1 deficient plants.

Phytochrome Associated PP2C (PAPP2C), another member of the same subfamily, was identified as a phosphatase interacting with phytochrome signaling. The results of this study implicated an indirect regulation of the phytochrome interacting factor 3 (PIF3) and thereby a function in Arabidopsis light signaling (Phee et al., 2008). Later, the same protein phosphatase was shown to control SA-dependent hypersensitive response (HR) to powdery mildew (Wang et al., 2012). PAPP2C interacts with the atypical resistance protein Resistance to Powdery Mildew 8.2 (RPW8.2). Protein levels of the PP2C increase upon infection and also after exogenously providing SA. Down-regulation of the PP2C increases resistance to the pathogen, making it a negative regulator of SA-dependent basal defense (Wang et al., 2012). Moreover, the involvement of two orthologous genes from maize in abiotic stress was examined using transgenic plants. In Arabidopsis, overexpression of ZmPP2C1 interferes with ABA-mediated responses to salt and drought (Liu et al., 2009). Ectopic expression of ZmPP2C2 enhances low temperature tolerance traits in tobacco (Hu et al., 2010). The molecular mechanisms underlying the effects observed remain to be elucidated.

Clade A

The first proteins of this subfamily have been identified in mutant screens for abscisic acid insensitive (*abi*) phenotypes. Among those mutants were *abi1* and *abi2* plants, which are severely impaired in the three classical physiological responses to ABA, i.e. inhibition of germination, inhibition of root growth and the closure of the stomates (Finkelstein and Somerville, 1990; Koornneef et al., 1984). Eventually, the *abi1-1* mutation, giving rise to these pleiotropic phenotypes, was identified as a hypermorphic allele of a PP2C gene (Leung et al., 1994). The corresponding protein ABI1, and its closely related homolog ABI2, are highly similar and control the full range of ABA responses (Leung et al., 1997; Meyer et al., 1994; Rodriguez et al., 1998a). Extensive genetic, biochemical and molecular studies in the ensuing years has deepened the understanding of the central function of the ABI1 and ABI2 protein phosphatases in ABA signal transduction (Gosti et al., 1999; Himmelbach et al., 2002; Meinhard et al., 2002; Sheen, 1998; Wu et al., 2003). The mutations responsible for the insensitivity, are a single amino acid exchange in the phosphatase domain, namely G180D in *abi1-1* and G168D in *abi2-1*. Interestingly, these substitutions decrease enzymatic activity (Bertauche et al., 1996; Leube et al., 1998; Leung et al., 1997). Later reports on other clade A PP2Cs supported their important function in ABA signaling. Two genes with remarkable Homology to ABI1/ABI2 (HAB1 and HAB2) were identified. Gain-of-function and knock-out phenotypes confirmed the negative regulation of ABA-dependent traits (Robert et al., 2006; Rodriguez et al., 1998b; Saez et al., 2004). There was also first evidence that a negative feedback-loop induced PP2C expression in the course of stress response (Leonhardt et al., 2004; Merlot et al., 2001). Additional family members were characterized

in a screen for mutants with ABA-Hypersensitive Germination (AHG). Both AHG1 and AHG3 are important mediators of ABA-dependent inhibition of germination (Nishimura et al., 2007; Nishimura et al., 2004; Yoshida et al., 2006b). The AHG3 allele is equivalent to PP2CA, which had been characterized as a strong negative regulator of ABA-dependent responses (Kuhn et al., 2006; Tahtiharju and Palva, 2001). Furthermore, a direct link between PP2C action and the control of cellular targets such as the Arabidopsis inward-rectifying Potassium Transporter 3 (AKT3) or the Slow Anion Channel 1 (SLAC1) was established (Lee et al., 2009; Vranova et al., 2001). Hence, PP2Cs were proven to be directly involved in ion-homoeostasis, which is particularly important for the regulation of stomatal closure. Accordingly, another member of this subfamily was identified as an interactor of the potassium transporter AKT1. It was named AKT1 Interacting PP2C 1 (AIP1) (Lee et al., 2007). Potassium influx is directly influenced by the activity of this PP2C (Cherel et al., 2002).

At the transcriptional level, clade A PP2Cs are concordantly regulated. Their expression is markedly induced by drought, osmotic and salinity stress as well as by exogenous ABA (Chan, 2012; Choudhury and Lahiri, 2011; Huang et al., 2007; Huang et al., 2008; Kreps et al., 2002; Seki et al., 2002). This transcriptional up-regulation results in negative feedback control of the ABA-dependent signal transduction. Investigation of additional ABA signaling mutants, completed the subset of clade A phosphatases by annotating three of the genes as Highly ABA-Induced PP2Cs (HAI1 - HAI3). HAI2 and AIP1 turned out to be the same gene. The expression levels of the three HAIs are strongly induced by exogenous ABA (> 50-fold). At the same time, their expression is greatly reduced in ABA signaling mutants (Fujita et al., 2009). Due to genetic redundancy, single knock outs give no or only weak phenotypes (Saez et al., 2006). Exceptions, like the strong ABA hypersensitivity in germination caused by a knockout of AHG1, can be explained by the corresponding expression patterns. While many of the related PP2Cs are not as abundant in mature seeds and in the early embryo, high levels of AHG1 transcript are predominantly found in these organs (Nishimura et al., 2007). In this case, redundancy is reduced. Taken together, the whole subfamily of clade A PP2Cs seems to be involved in the central control of ABA-dependent responses.

1.4 Downstream factors of ABA response regulating PP2Cs

1.4.1 Ion channels and other targets

As already mentioned, ion channels constitute a subset of direct or indirect targets of PP2Cs. Ion homeostasis is fundamentally important for the hydrostatic properties of the plant cell, the nutritional status and physiological processes. Potassium transporters such as AKT1, AKT3 and KAT1 are functionally controlled by phosphorylation (Cherel et al., 2002; Lee et al., 2007; Sato et al., 2009;

Xu et al., 2006). Similarly, S-type anion channel SLAC1 and SLAC1 Homolog 3 (SLAH3) are tightly regulated in a phosphorylation dependent manner (Geiger et al., 2011; Geiger et al., 2009; Schmidt et al., 1995; Xue et al., 2011). In particular, stomatal opening and closing events are governed by ionic fluxes. Thus, the guard cell provides basic insights into ion channel regulation based on PP2C action (Lee et al., 2009; Sirichandra et al., 2009b; Xue et al., 2011).

Besides ion channels, a plethora of PP2C interacting proteins has been identified using different approaches. This set of versatile interaction partners includes many genes of unknown function. Yet, also some genes with a known, specific function interact with one or more clade A phosphatases, as exemplified by the interaction data collected in the BioGrid database (Chatr-Aryamontri et al., 2013; Stark et al., 2006). For instance, Glutathione Peroxidase 3 (GPX3) was shown to interact with ABI1 and ABI2, providing a link between ABA signaling and cellular redox state (Miao et al., 2006). ABI2, but not ABI1, physically interacts with the preprotein of fibrillin, which is involved in photoprotection (Yang et al., 2006). HAI2 and HAI3 were found to interact with At3g06610, a DNA-binding Enhancer-related protein. This might hint towards a function in transcriptional regulation (Consortium, 2011). Palpably, each PP2C has additional features besides their common function in the core ABA signaling module.

1.4.2 Transcriptional regulators

Indeed, PP2Cs are part of the machinery controlling ABA-dependent gene regulation. Plenty of TFs from different families have been implicated in the vast transcriptome alterations caused by ABA (Fujita et al., 2011; Rushton et al., 2012). A characteristic feature of a multitude of ABA-responsive genes is the presence of a conserved *cis*-regulatory element in their promoter regions (Hobo et al., 1999). The so-called ABA Response Element (ABRE) consists of the core sequence ACGTG$\frac{C}{T}$C (Hattori et al., 2002). The group of ABRE Binding Proteins (AREBs), or synonymously termed ABRE binding factors (ABFs), specifically target this motif and exert gene regulatory function (Kang et al., 2002; Uno et al., 2000). These transcription factors belong to the group of basic region/leucine zipper motif (bZIP) transcription factors (Jakoby et al., 2002). The ABF family contributes extensively to the substantial ABA-dependent transcriptional reprogramming (Kim et al., 2004a; Lopez-Molina et al., 2002; Nakashima et al., 2006; Sharma et al., 2011; Wang et al., 2013; Yoshida et al., 2010). Peculiarly, the bZIP TFs are regulated by phosphorylation (Hong et al., 2011; Kobayashi et al., 2005; Muniz Garcia et al., 2012). Again, PP2Cs from clade A serve as negative regulators by influencing the ABF phosphorylation status. This is achieved either through direct catalysis (Lynch et al., 2012) or indirectly through inhibition of the activating kinases (Fujii et al., 2007; Fujita et al., 2013). Besides

targeting ABFs, there is evidence that clade A PP2C also act on other TFs. Homeobox protein 6 (HB6), a member of the plant specific homeodomain-leucine zipper (HD-Zip) family of TFs (Schena and Davis, 1992), is binding to ABI1 and functionally involved in ABA response (Himmelbach et al., 2002).

1.4.3 Kinases

The large family of protein kinases plays a major role in signal transduction and other regulatory mechanisms. Protein kinases function as mutual antagonists of phosphatase action and are able to evoke diverse physiological, molecular and biochemical processes. ABA-dependent stress signaling is to a large extent mediated by a variety of protein kinases from different subfamilies.

Calcium-dependent protein kinases (CPKs)

Ubiquitously, Ca^{2+} serves as a second messenger in eukaryotic cellular signaling. Also, ABA signal transduction includes Ca^{2+}-mediated responses (Kiegle et al., 2000; Knight et al., 1997; Schroeder et al., 2001). One subfamily of protein kinases, namely the CPKs, is directly regulated by Ca^{2+} ions. The 34 Arabidopsis CPKs possess a calmodulin-like domain containing one or more EF-hands, i.e. specific Ca^{2+}-binding sites. In addition to the Ca^{2+}-binding elements, this group features a kinase domain, a variable N-terminus and a pseudosubstrate-like autoinhibitory domain (AID) (Cheng et al., 2002). In Arabidopsis, CPK4 and CPK11 control ABA-dependent traits. These two protein kinases are also able to phosphorylate and activate ABFs, directly linking Ca^{2+}-signal integration to transcriptional orchestration (Zhu et al., 2007). Accordingly, CPK10, CPK21, CPK23 and CPK32 were shown to contribute to ABA-dependent physiological responses (Choi et al., 2005; Franz et al., 2011; Ma and Wu, 2007; Zou et al., 2010). Overexpression of CPK6 mediates increased tolerance to drought and salt stress in transgenic Arabidopsis lines (Xu et al., 2010). For some of the CPKs relevant for abiotic stress signal transduction, it is known that there is a close regulatory relation with the PP2Cs from clade A. The aforementioned CPK6 controls SLAC1 activity. This is antagonized by PP2C function (Brandt et al., 2012). The same is true for CPK21 in SLAC1 regulation (Geiger et al., 2010). In the case of SLAH3, even a direct regulation by protein kinase-phosphatase pairing is reported (Geiger et al., 2011). Lynch et al. (2012) have depicted a complex network of direct interaction and mutual control of downstream targets for CPK11 and clade A PP2Cs. Depending on the interaction affinities between CPK11, clade A PP2Cs and/or ABFs, the activity of the protein kinase and thus ABF phosphorylation status, are controlled in a PP2C-dependent manner. A similar mode for negative regulation of Calmodulin/Ca^{2+}-dependent protein kinases (CaMKs) is observed in the mammalian system. CaMK phosphatase (CaMKP) PP2C isoforms directly dephosphorylate the protein kinases corresponding to

a "switch-off" mechanism (Ishida et al., 2008; Sueyoshi et al., 2012; Tada et al., 2006). Moreover, there is an overlap of CPK subsets involved in abiotic and biotic stress signaling (see Fig. 4). CPK4, CPK6 and CPK11 are involved in early response to PAMP-triggered defense reactions (Boudsocq et al., 2010; Kobayashi et al., 2007; Munemasa et al., 2011). This function is partly mediated by the crucial control of ROS production. The function of CPK1 is linked to ROS production and SA-mediated systemic resistance (Coca and San Segundo, 2010). Defense signaling connected to herbivory is controlled by CPK3 and CPK13 (Kanchiswamy et al., 2010). Hence, CPKs are central integrators of diverse stress signaling cascades.

Another subset of kinases interwoven in abiotic stress signaling are the sucrose non-fermenting 1 (SNF1) related protein kinases (SnRKs). Based on homology and conserved domain architecture this group is subdivided in three families (1-3) (Halford and Hardie, 1998; Hrabak et al., 2003). The SnRK1 subfamily proteins are part of the sugar and energy starvation pathways in eukaryotes (Hardie, 2007; Mihaylova and Shaw, 2011). In addition, the few Arabidopsis SnRK1s significantly influence and coordinate hormone-based developmental and environmental traits, including ABA-specific responses (Baena-Gonzalez et al., 2007; Jossier et al., 2009; Radchuk et al., 2010). SnRK2 and SnRK3 subfamilies directly take part in the ABA signal transduction pathways.

SnRK3s

SnRK3 proteins are equivalent to the group of Calcineurin B-like (CBL)-interacting protein kinases (CIPKs) in Arabidopsis (Hrabak et al., 2003). CBLs are a plant-specific family of Ca^{2+} sensory proteins, which directly bind Ca^{2+}. Their expression pattern is responsive to environmental stimuli such as high salt or drought stress. The CBLs are involved in Ca^{2+}-mediated responses (Kudla et al., 1999; Liu and Zhu, 1998) by interacting with coupled signaling effectors, i.e. the CIPKs. Each CBL cooperates with a subset of CIPKs to mediate phosphorylation of target factors (Jeong et al., 2005; Kim et al., 2000; Nozawa et al., 2003). Conformational changes in the CBL upon Ca^{2+} binding induce pairing (Gong et al., 2004). The specific interaction of the sensor and the protein kinase is established via the conserved CBL-interaction domain (CID) in the CIPKs (Akaboshi et al., 2008; Albrecht et al., 2001) (see Fig. 4). A complex network of CBLs and matching CIPKs achieves Ca^{2+}-signal integration, and yet selective regulation of downstream processes (Batistic and Kudla, 2004; Kolukisaoglu et al., 2004). Among the physiological substrates of CIPKs, there is for example, the AKT1 potassium channel (Li et al., 2006; Xu et al., 2006) (see also 0). The positive regulation of AKT1 by CIPK23 provides an essential link from Ca^{2+} to ABA signaling (Cheong et al., 2007). Moreover, this activation of ion channel

function is controlled by the counteracting clade A PP2Cs (Lan et al., 2011; Lee et al., 2007). But CBLs not only bind Ca^{2+} but also define functional characteristics due to their localization (Batistic et al., 2010). CIPK6 is an important factor of salt tolerance and development (Tripathi et al., 2009; Tsou et al., 2012). It requires CBL targeting for proper function on its target AKT2 (Held et al., 2011). Independent of their connection to ABA signal transduction through the regulation of important ion channels, CIPKs were identified as clade A phosphatase interacting proteins. CIPK24 was characterized as one of the central factors in the hypersaline stress response. The protein kinase, alias Salt Overly Sensitive 2 (SOS2), was shown to be activated by Salt overly sensitive 3 (SOS3/CBL4) (Guo et al., 2001; Halfter et al., 2000). The protein kinase regulates vacuolar Ca^{2+} flux, for instance, via controlling Cation Exchanger 1 (CAX1) function (Cheng et al., 2004). ABI2 was found in a screen for CIPK24 interacting proteins. In addition, Y2H analyses showed interaction of ABI2 with CIPK8 and CIPK15 as well as of ABI1 with CIPK20, CIPK24 and CIPK15 (Guo et al., 2002; Ohta et al., 2003). Also AHG1 and PP2CA are physically connected to CIPKs. The collected data imply a model with a direct mechanistic association of CBLs, CIPKs, PP2Cs and the respective signaling targets (Lan et al., 2011). A set of CIPKs seems to be involved in stress signaling apart from the PP2C signaling relay. CIPK1 and CIPK8 control important ABA-dependent and -independent traits (D'Angelo et al., 2006; Hu et al., 2009; Knutova, 2008).

CPKs	Signaling pathway (target)
4/11/32	Abiotic stress / ABA (ABFs)
6/21	Abiotic stress / ABA (SLAC1)
1	Defense / SA
4/6/11	Defense / PAMP (RbohB)
3/13	Defense / herbivory

CIPKs	Signaling pathway (target)
6	Salt stress / nutrition (AKT2)
1/8	Abiotic stress / nutrition
23	Abiotic stress / nutrition (AKT1)
24	Salt stress / nutrition (CAX1)

Fig. 4: Comparison of CPK and CIPK domain structure and function. The kinase and auto-inhibitory domains are common to both kinases. CPKs are directly activated upon Ca^{2+}-binding to the EF-hand containing calmodulin-like domain (CLD). This causes a conformational change releasing the catalytic site from AID-dependent inhibition. CIPKs are activated by CBL-interacting domain (CID)-mediated docking of a CBL Ca^{2+}-sensor protein. Examples for cellular function and molecular interaction of certain CPKs and CIPKs are provided (Boudsocq et al., 2010; Brandt et al., 2012; Cheng et al., 2004; Cheong et al., 2007; Choi et al., 2005; Coca and San Segundo, 2010; D'Angelo et al., 2006; Geiger et al., 2010; Held et al., 2011; Hu et al., 2009; Kanchiswamy et al., 2010; Kobayashi et al., 2007; Lee et al., 2007; Lynch et al., 2012; Tripathi et al., 2009; Xu et al., 2006; Zhu et al., 2007).

SnRK2s

SnRK2s, the third subgroup, contribute universally to ABA-dependent responses (Boudsocq et al., 2004). SnRK2.2 and SnRK2.3 were shown to be important components in controlling processes such as root growth, germination and transcriptional regulation (Fujii et al., 2007). The kinase Open Stomata 1 (OST1), equal to SnRK2.6, is responsible for proper stomatal control. Together with SnRK2.2 and SnRK2.3, it constitutes a prominent and redundantly acting clade of SnRK2s, which is vital for ABA signal transduction, especially in guard cells (Fujii and Zhu, 2009; Fujita et al., 2009; Nakashima et al., 2009; Sirichandra et al., 2009b). Even though other SnRK2 proteins contribute to abiotic stress response, the 2.2/2.3/2.6 subgroup appears particularly relevant for ABA-specific core signaling (Boudsocq et al., 2004; Fujita et al., 2009; Kulik et al., 2011; Umezawa et al., 2004). The molecular function of these protein kinases has been extensively investigated. Upon activation, a variety of cytosolic and nuclear proteins is targeted (Ng et al., 2011). SnRK2.6 connects ABA-elicited signaling via activation of the Arabidopsis Respiratory Burst Oxidase Homolog F (AtRbohF) to the production of ROS as secondary messenger (Sirichandra et al., 2009a). ROS have been known for a long time to be crucially involved in stress and ABA signaling (Foreman et al., 2003; Kwak et al., 2003; Mori and Schroeder, 2004; Overmyer et al., 2003). Activation of the Potassium channel in Arabidopsis 1 (KAT1) depends on specific phosphorylation by SnRK2.6 (Sato et al., 2009). The same holds true for SLAC1, which is tightly regulated by SnRK2.6 and antagonizing PP2Cs from clade A (Geiger et al., 2009; Lee et al., 2009). Crystallographic studies and molecular data provide evidence that this antagonism is due to the direct coupling of the phosphatase and the protein kinase. These prominent SnRK2s are inactivated through the physical constraint exerted by the protein phosphatases (Vlad et al., 2009; Xie et al., 2012; Yoshida et al., 2006a). Apart from cytosolic targets, SnRK2s mediate transcriptional regulation through phosphorylation-dependent activation of ABFs in the nucleus (Fujita et al., 2009; Kobayashi et al., 2005; Sirichandra et al., 2010). These master transcriptional regulators were shown to cooperatively regulate ABA-responsive gene expression (Yoshida et al., 2010). Major negative control to this signal cascade is again exerted by clade A PP2Cs (Umezawa et al., 2009; Xie et al., 2012). Variable interactions between SnRK2s and PP2Cs drive signaling outcome (Fujita et al., 2009; Hirayama and Umezawa, 2010).

1.5 Regulators of PP2C activity

In 2009, two groups independently identified a family of cytosolic ABA receptor proteins. Park et al. (2009) characterized the Pyrabactin Resistance 1 (PYR1) gene necessary for binding of the synthetic ABA agonist pyrabactin. In this chemical genetics approach, PYR1 and closely related PYR1-like (PYL) proteins were shown to be receptors for the phytohormone. ABA-dependent signal transduction is

achieved through direct inhibition of PP2C negative regulators (Park et al., 2009). At the same time, Ma et al. (2009) isolated two closely related proteins as PP2C interacting regulators. In the presence of ABA, the Regulatory Component of ABA Receptor (RCAR) inhibits protein phosphatase activity. The binding is specific and stereo-selective for the bioactive (S)-ABA enantiomer in most receptor complexes. Calculated values for 50 %-inhibitory concentration (IC_{50}) of the ligand are in the physiologically relevant, sub-micromolar range (Ma et al., 2009; Melcher et al., 2010a; Szostkiewicz et al., 2010).

1.5.1 The receptor family and related proteins

The RCAR family consists of 14 genes in *A. thaliana*. Based on sequence homology, three subgroups were defined (Fig. 5 A). These proteins with a size of approximately 20 kDa are related to the major birch pollen allergen of *Betula verrucosa* 1 (Bet v 1) protein family. They share the characteristic fold of seven antiparallel β-sheets wrapped around a long central α-helix and one or two smaller vicinal helices (Gajhede et al., 1996) (see Fig. 5B).

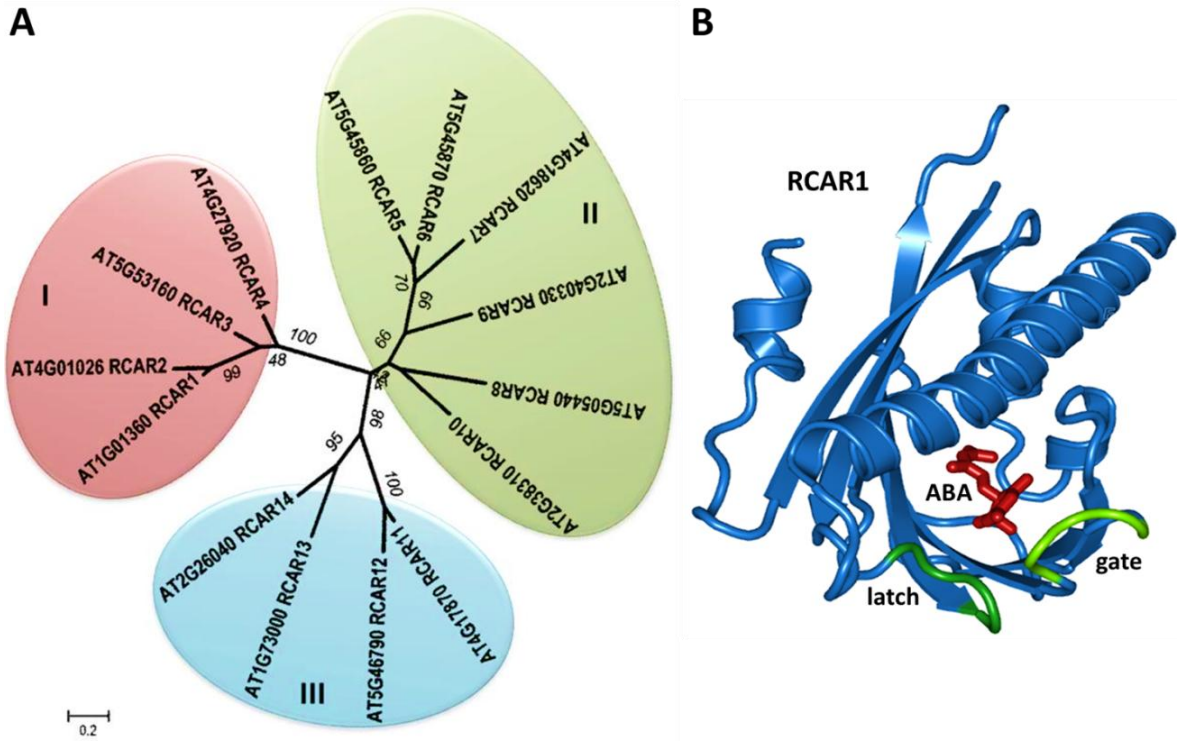


Fig. 5: Phylogenetic tree and characteristic fold of the RCAR family. (A) The unrooted Maximum Likelihood-tree was corrected using the Dayhoff model (Schwartz and Dayhoff, 1978). Numbers indicate bootstrapped frequencies in 500 replicates (Felsenstein, 1985). Branch lengths represent evolutionary distance and are measured in the number of amino acid substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). Subgroups are labeled according to Ma et al. (2009). (B) The structure of ABA-bound RCAR1 derived from Protein Data Bank (PDB-code: 3OQU) is illustrated as cartoon using PyMol (Schrodinger, 2010). ABA is presented in stick mode and highlighted in red. The gate and latch loops are highlighted in green.

The typical Bet v 1 fold is found, throughout the angiosperms, also in the pathogenesis-related subfamily 10 (PR-10) proteins and the major latex proteins (MLPs) (Liu and Ekramoddoullah, 2006; Nessler et al., 1990; Osmark et al., 1998). On the basis of structural characteristics, Bet v 1 and its relatives were designated to bind a number of hydrophobic ligands (Marković-Housley et al., 2003; Mogensen et al., 2002). The PR-10 Cytokinin-Specific Binding Protein (CSBP) from *Vignia radiata*, for example, showed high affinity binding to cytokinins in the low micromolar range (Pasternak et al., 2006). The binding of synthetic cytokinins was also found for a *Lupinus luteus* PR-10 homolog (Fernandes et al., 2009; Fernandes et al., 2008). An analogous domain, alternatively termed Star-related lipid transfer (START) domain was shown to be involved in lipid binding. For example, the human START proteins are involved in the transport of steroids (Clark, 2012). Interspecies comparison proved a widespread distribution of this conserved helix-grip-fold across kingdoms and in different signaling pathways (Iyer et al., 2001; Radauer et al., 2008).

1.5.2 Function of RCAR-related Major Latex Proteins

MLPs are closely related to RCARs. They share the common helix-grip-fold, forming a cavity for ligand binding. Structural and binding data derived from the investigation of MLP28 confirmed this proposed function. The MLP engulfed progesterone, a human steroid hormone resembling the brassinolide phytohormone from plants (Lytle et al., 2009). Yet, there is no specific implication or molecular mechanism known for the function of MLPs in the plant. Transcriptomic analyses indicated a role for two MLPs in the response to gravitropic stimulation (Kimbrough et al., 2004). Another report assigned two other MLPs a function in the regulation of vegetative growth and bolting (Guo et al., 2011). The transcript levels of the two MLP genes, termed cis-Cinnamic acid Enhanced 1 (ZCE1) and ZCE2 increased specifically in response to the substance cis-cinnamic acid. By an unknown mechanism these proteins influence flowering time in Arabidopsis. Also in seed dormancy control, several MLPs were identified as important factors (Chibani et al., 2006). Overexpression of the MLP28 homolog from cotton desensitizes transgenic Arabidopsis to salt stress-mediated stunting of germination (Chen and Dai, 2010). Aside from being involved in developmental cues, several MLPs are engaged in defense signaling (Jones et al., 2006; Sun et al., 2010). For instance, two MLP genes were significantly down-regulated in plants infected with tobacco mosaic virus (TMV) (Golem and Culver, 2003). A subset of MLPs was also differentially expressed after PAMP perception or during infection (Ascencio-Ibanez et al., 2008; Devos et al., 2006; Siemens et al., 2006).

1.5.3 Molecular mode of RCAR function

The identification of the soluble receptor proteins allowed insights into the molecular mechanism of PP2C inhibition. There are a number of amino acid residues relevant for the selective coordination of the ligand and for accurate docking to the PP2C. In the presence of ABA, two conserved loop structures (β3-4 and β5-6) undergo conformational changes (Nishimura et al., 2009; Santiago et al., 2009a). These loops enclose the ligand molecule and were therefore termed "gate" and "latch" (Melcher et al., 2009). Analysis of RCAR variants with impaired ligand coordination, demonstrated accessory disruption of PP2C interaction (Nishimura et al., 2009). ABA binding-induced closure of the RCAR loops, generates the compatible interface enabling the assembly of the trimeric complex with the phosphatase (Yin et al., 2009). Ultimately, the docking of the PP2C "locks" the closed state of the RCAR sensor and markedly increases the ligand binding affinity. A conserved, protruding Trp-residue (W300 in ABI1) traps the receptor loops and interacts with the ABA molecule itself (Melcher et al., 2009; Miyazono et al., 2009). Only the holoreceptor complex displays binding affinities in the sub-micromolar, and hence the physiologically relevant, range (Ma et al., 2009; Santiago et al., 2009b; Szostkiewicz et al., 2010).

Combinatorial concurrence of different RCARs and clade A PP2Cs was analyzed using the Y2H-system, *in vitro* and *in vivo*. In yeast, the interaction with HAB1 was strictly ABA-dependent for subgroup III RCARs and RCAR10. The other RCARs investigated showed interaction without exogenously supplied ABA (Park et al., 2009). A similar pattern of interaction was shown for the HAI-PP2Cs (Bhaskara et al., 2012). The assembly of the regulatory complex in Y2H analyses was greatly reduced in *abi1-1* and *abi2-1* mutants, compared to the plants harboring the wt phosphatases (Ma et al., 2009; Szostkiewicz et al., 2010) (see also Fig. 6A). These results finally disclosed the function of the mutated PP2Cs. Even though the point mutation decreases enzymatic activity of the phosphatases, they have a gain-of-function effect on ABA signal transduction (Bertauche et al., 1996; Gosti et al., 1999). The docking of the RCARs to the phosphatase is greatly extenuated, due to the steric hindrance caused by the exchange of the relevant amino acid (Miyazono et al., 2009; Santiago et al., 2012). The regulation of the receptor complex has been extensively analyzed also *in vitro*. The assembly is predicated on a 1:1 stoichiometry of RCAR and PP2C (Fig. 6B). Different combinations feature different binding affinities – for the binding of the RCARs to the PP2Cs on the one hand, and for the binding of the ligand on the other hand (Dupeux et al., 2011; Santiago et al., 2009b; Szostkiewicz et al., 2010) (Fig. 6C).

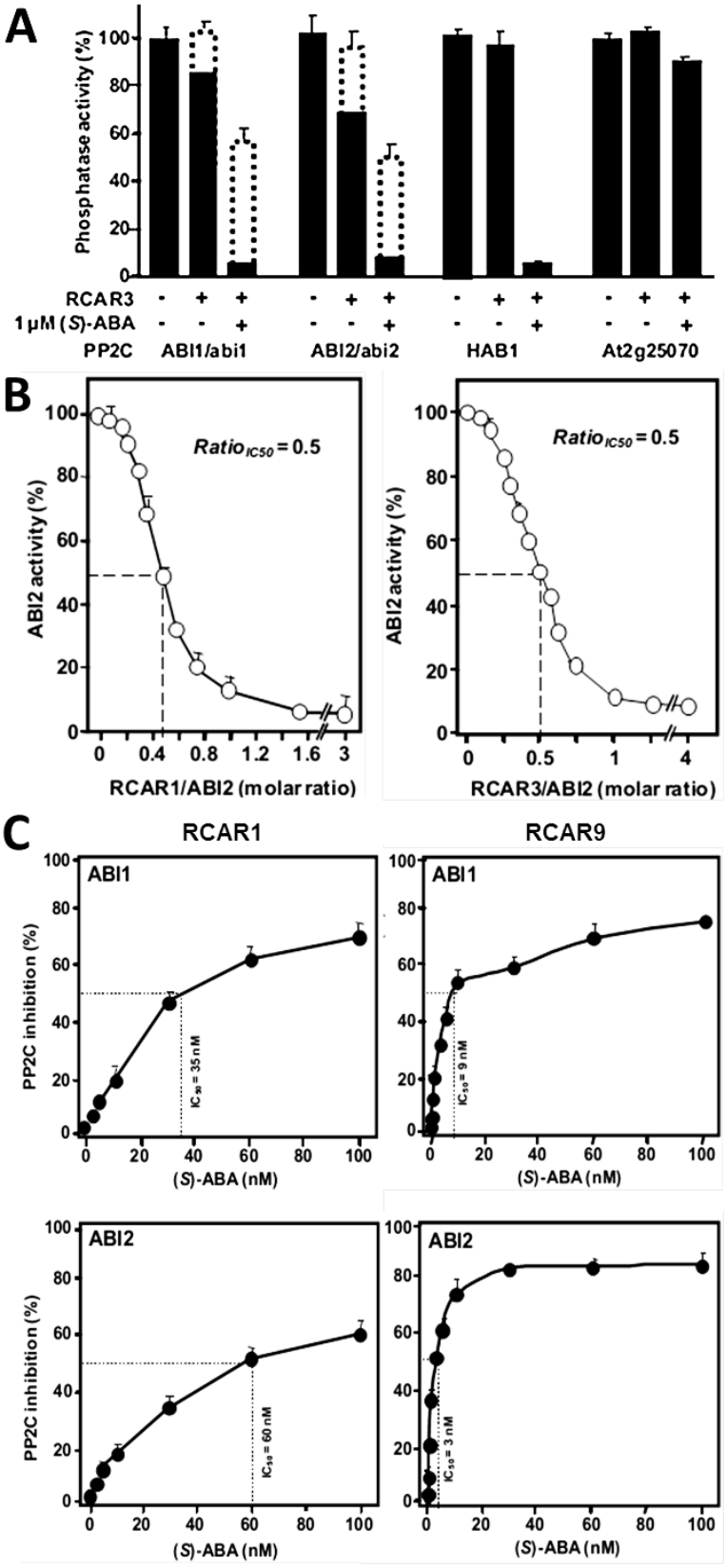


Fig. 6: Biochemical characteristics of different RCAR-PP2C combinations. (A) RCAR3- and ABA-dependent inhibition of ABI1, ABI2 and HAB1. The PP2C At2g25070 serves as a negative control. The mutant versions *abi1* and *abi2* are partially resistant to ABA/RCAR-mediated inhibition (dotted lines). (B) Stoichiometric calculations exhibit a 1:1 RCAR:PP2C ratio. (C) Calculation of ligand IC_{50} values for different co-receptor complexes, of PP2Cs ABI1 or ABI2 with RCARs 1 or 9, based on ABA-dependent PP2C inhibition. Modified from Szostkiewicz (2010)

The regulation of RCAR-mediated signaling was also investigated *in vivo*. Fujii et al. (2009) reconstituted the complete ABA signaling cascade using a minimal set of components. The use of four essential modules, namely RCARs, PP2Cs, SnRK2s and ABFs, is enough to provoke ABA-dependent signaling in Arabidopsis protoplasts. This relatively small set of modules emerged as the core of a signaling cascade, ranging from hormone perception to gene regulation (Cutler et al., 2010). In the plant, the situation is more complex because of temporarily and spatially diverging expression patterns (Antoni et al., 2013; Gonzalez-Guzman et al., 2012; Szostkiewicz et al., 2010). Nevertheless, the interaction of clade A PP2Cs and the RCAR proteins is confirmed *in planta*. RCARs were identified as major PP2C interacting proteins (Nishimura et al., 2010). Also, several PP2Cs were co-purified with a tagged version of RCAR3, implying *in vivo* interaction (Antoni et al., 2013). Furthermore, the analysis of single and multiple knockout lines of either RCARs or PP2Cs, further substantiated the vital role of the co-receptor complex. Genetic redundancy amongst RCAR, PP2C, SnRK2 and ABF family members impeded phenotypic analysis. There are only a few instances of single knockout mutants exhibiting non-redundant phenotypes. RCAR3, for example, is explicitly important for root ABA signaling (Antoni et al., 2013). An AHG1 knockout distinctively impairs germination traits (Nishimura et al., 2007). An elimination of SnRK2.6 function derails stomatal closure in response to drought (Assmann, 2003). Apart from these examples, only the concomitant removal of redundant proteins gives rise to substantial phenotypes (Bhaskara et al., 2012; Fujita et al., 2009; Gonzalez-Guzman et al., 2012; Park et al., 2009; Rubio et al., 2009; Saez et al., 2006; Yoshida et al., 2010). Contrarily, the overexpression of one of the components is sufficient to impair the signaling outcome (Antoni et al., 2012; Lee et al., 2012; Santiago et al., 2009b).

1.6 Molecular mimicry in PP2C function

The structural data gained from RCAR-PP2C complexes elucidated the processes underlying ABA-dependent signaling. The interface of RCARs and PP2Cs comprises a number of conserved amino acids (Hao et al., 2011; Melcher et al., 2010a). For example, in virtually all RCAR family members these important residues are present (Mosquna et al., 2011; Shibata et al., 2010). These conserved sites in the regulatory component and the PP2C, constitute a meshwork of crucial molecular interactions (Peterson et al., 2010). Surprisingly, the same grid of interacting amino acids is largely conserved not only in the RCAR proteins, but also in one of the substrates, i. e. the SnRK2 protein kinases (Ng et al., 2011; Yunta et al., 2011). The structural analyses revealed a set of conserved residues that is vital for the interaction of RCARs and SnRK2s and the PP2Cs (Soon et al., 2012a; Xie et al., 2012).

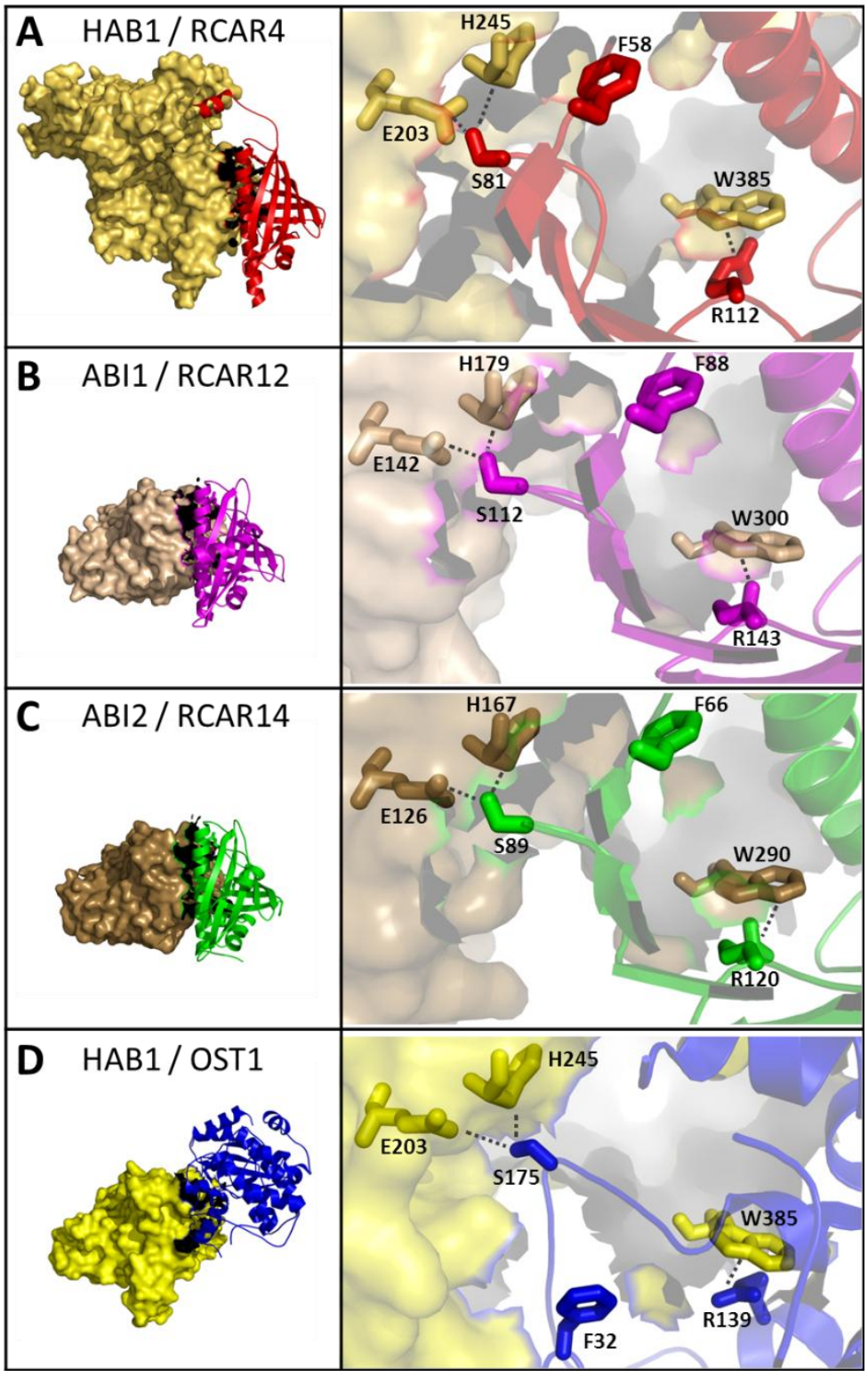


Fig. 7: Molecular mimicry in PP2C signaling. Different complexes of PP2Cs and their interacting proteins RCARs or SnRK2.6 are depicted in total and close-up views (left and right panels, respectively). Three important amino acids are presented in stick mode in the PP2C (brown/yellow) and either RCARs (red, magenta, green) or SnRK2.6 (blue). The catalytic site of the PP2Cs features conserved E, H and W residues. The pseudosubstrate RCAR exposes conserved F, S and R residues. Similarly, SnRK2.6/OST1 possesses a conserved F, R and S grid. The crystal structures of HAB1/RCAR4 (A) (Hao et al., 2011), ABI1/RCAR12 (B) (Miyazono et al., 2009), ABI2/RCAR14 (C) and HAB1/OST1 (D) (Soon et al., 2012a) are illustrated using PyMol (Schrodinger, 2010) (PDB codes: 3RT0, 3JRQ, 3UJL, 3UJG, respectively). Dotted lines indicate molecular interaction of the corresponding residues. Modified from Fuchs et al. (2012).

Fig. 7 illustrates the overlap in PP2C binding interfaces between RCARs (Fig. 7 A-C) and the SnRK2 OST1 (Fig. 7D). The catalytic cleft of the PP2Cs is structurally highly conserved in clade PP2Cs. RCARs as well as SnRK2s, are accurately clinched at the same site of the PP2C, i.e. the active center. The similarity between the substrate SnRK2 and the regulator RCAR (indicated by the residues highlighted) was interpreted as molecular mimicry (Soon et al., 2012a). In both cases, ionic interactions are established by the conserved S and R residues. Additionally, the F residue in close vicinity stabilizes the complex through hydrophobic interaction. An analogous system of substrate simulation is known well studied for pathogenic interference during immune response (Chan et al., 2009; Price et al., 2009). This kind of regulation of the PP2C by the RCAR pseudosubstrate resembles the autoinhibitory control of multi-subunit protein kinases (Ha et al., 2012; Harper et al., 1994; Scott et al., 2007) (compare Fig. 4).

1.7 ABA signaling

In conclusion, the present molecular mechanism of ligand-dependent inhibition of a negative regulator constitutes a new paradigm in the control of stress signal transduction. The current model, including the major players in ABA dependent signal transduction, is depicted in Fig. 8.

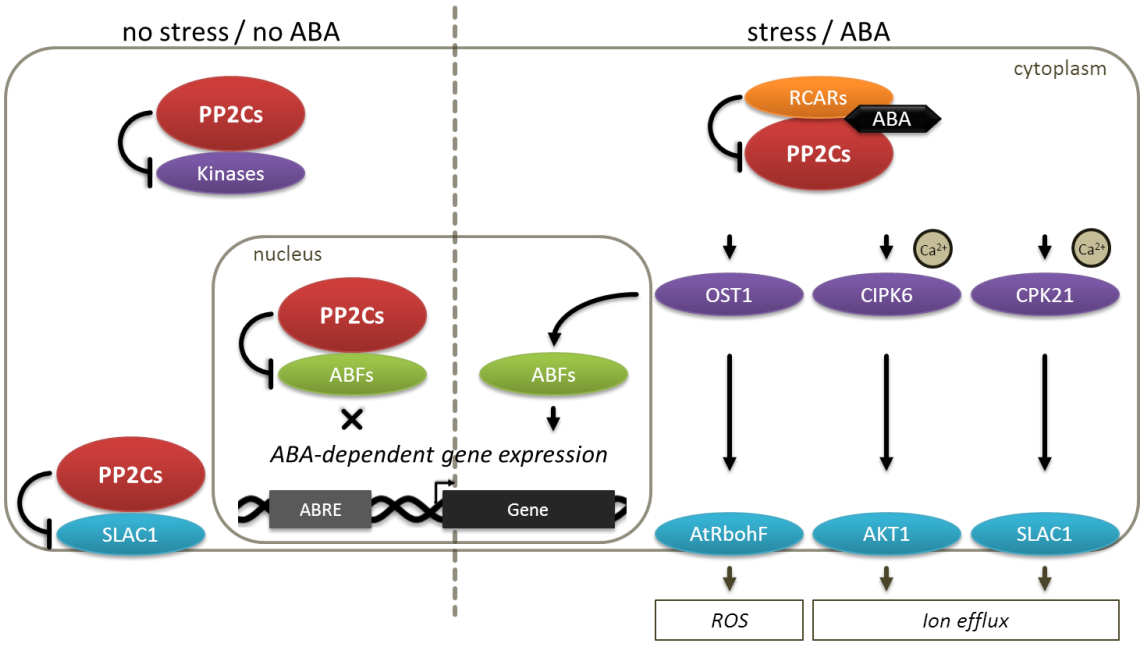


Fig. 8: Model of cellular ABA signal transduction. In the absence of the stress stimulus, PP2Cs block the function of downstream factors (kinases, TFs, other targets colored in purple, green and cyan respectively). Under stress conditions, the RCAR-ABA-PP2C receptor complex is formed. Downstream factors are released from PP2C-dependent inhibition. ABA-dependent cellular processes (e.g. ROS production, ion efflux) are effected, partly involving Ca^{2+} secondary signals. In the nucleus, ABA-dependent transcriptional modulation is activated.

1.8 The aim of this work

Protein phosphatases 2C have evolved as central regulators of stress response in evolution. Particularly in plants, PP2C are controlling a variety of elementary signaling pathways. The investigation of the molecular mechanisms governing these processes is essential to complete our understanding. The direct targets and the regulatory factors of these phosphatases are largely unknown. The regulatory mechanism of PP2C deactivation by RCAR receptor proteins in a ligand-dependent manner is a new paradigm. The PP2C-dependent regulation of transporters, transcription factors and other substrates might be transferable to other signaling cascades in the plant.

In this work, the challenge was to characterize the PP2C-related regulation of ABA signaling. The framework of PP2C substrates, partners and regulators was to be analyzed in yeast and in the protoplast system. The effect of newly identified components on global ABA signaling was examined. Accordingly, the physical interaction of PP2Cs and ABA-related protein kinases was investigated. Eventually, a regulation of PP2C function analogous to the mechanisms in ABA signal transduction was studied for other stress response pathways.

2 RESULTS

2.1 PP2C-RCAR interaction

2.1.1 Complex formation in yeast

The identification of the family of soluble ABA receptors, namely the RCAR proteins, and their docking to the ABA response-regulating PP2Cs uncovered the main molecular switch of this important signaling pathway (Ma et al., 2009; Park et al., 2009). Genetic redundancy of the protein families impeded the investigation of the ABA perception network (Bhaskara et al., 2012; Gonzalez-Guzman et al., 2012).

To elucidate the mechanisms governing the assembly of different co-receptor complexes, the complement of all possible PP2C-RCAR-interactions was to be analyzed in the heterologous Y2H-system. Therefore the protein coding sequences (CDS) of the proteins to be tested were cloned into pGAD424 and pBridge vectors of a Galactose Metabolism 4 (GAL4) TF-based two-hybrid system (Bai and Elledge, 1996). The resulting constructs encoded in-frame GAL4 TF activation domain (AD)-fusions with the nine PP2Cs (pGAD) or GAL4 TF DNA binding domain (BD)-fusions with the 14 RCAR proteins (pBridge). Plasmid DNA extracted from positive bacterial clones was sent for sequencing to GATC (GATC Biotech AG, Cologne, Germany). AD and BD constructs with correct sequences were selected and sequentially transformed into AH109 yeast strain (James et al., 1996). Double transformants were assayed for reporter activation. The selection of positive transformation is achieved through Leu and Trp autotrophy genes encoded on pGAD and pBridge plasmids, respectively. Interaction of the candidate proteins reestablishes a functional GAL4 TF leading to transcriptional activation of the reporter genes (Gietz et al., 1997). The induction of one of these reporters, namely histidine (His) biosynthesis, allows growth on medium lacking this amino acid. Combinations exerting no interaction will not proliferate on triple selective medium (-L/-W/-H).

To get an overview of the possible interactions, the growth of the transformed yeast lines carrying all the conceivable vector combinations was monitored on selective media as a read-out. ABA was shown to stabilize the interaction of some RCARs with PP2Cs in yeast (Park et al., 2009), so the assay was performed with or without the ligand in the media. Fig. 9 shows examples of these assays with the nine clade A PP2Cs and one non-clade A PP2C as a control. They were tested against RCAR3, RCAR7 and RCAR11 representing the receptor subfamilies I, II and III, respectively.

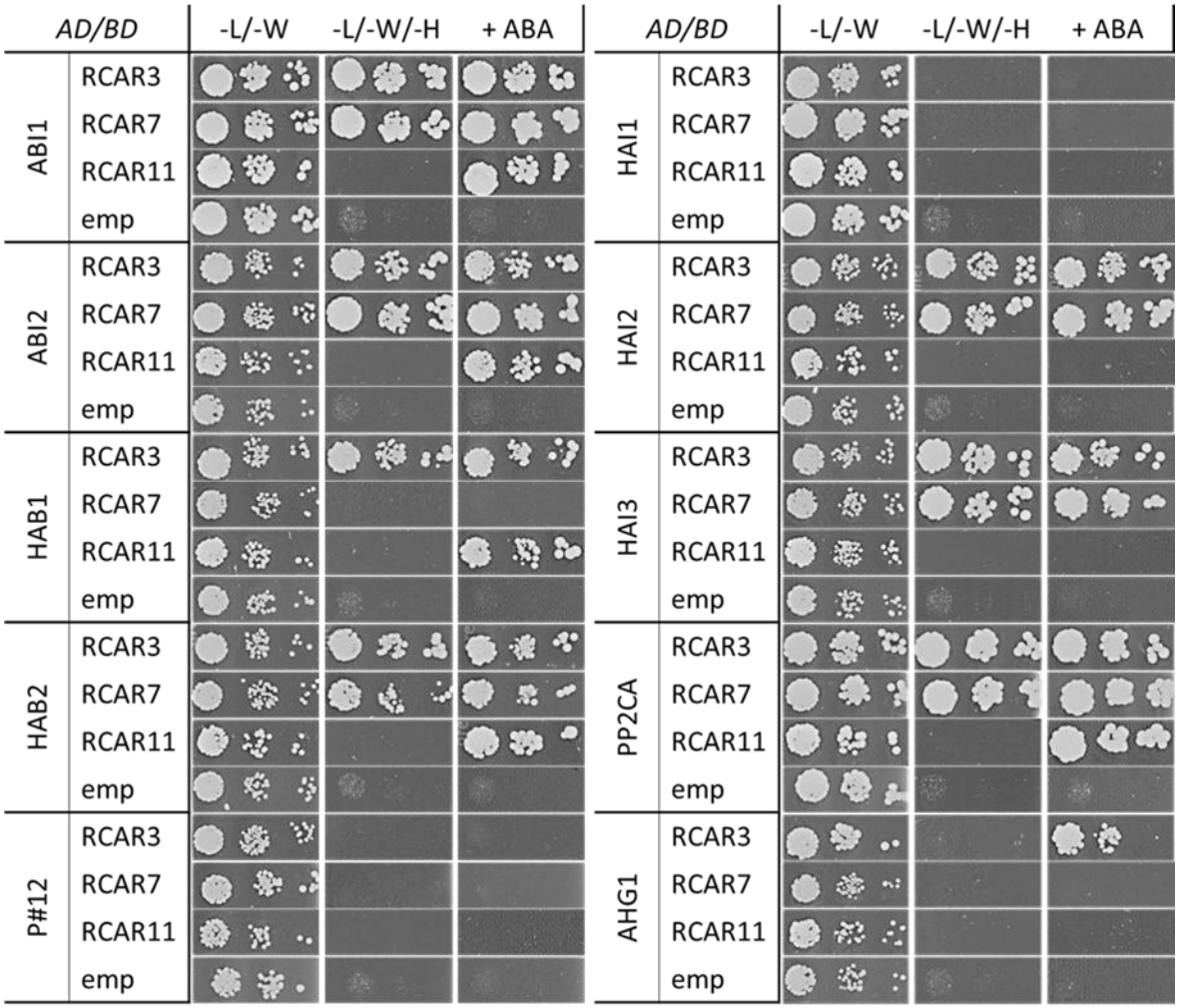


Fig. 9: Interaction of PP2Cs and RCARs in the Y2H-system. Exemplary combinations of clade A PP2Cs and RCAR3, RCAR7 and RCAR11 are spotted as dilution series. The yeast cells were selected for His-autotrophy as a read-out for interaction. Growth on double selection (-L/-W) reflects positive dual transformation and growth on triple selection (-L/-W/-H) reflects positive interaction. Ligand dependency is monitored in triple selective medium supplied with 30 µM ABA (+ ABA). Combinations of PP2C#12 (P#12) and empty vectors (emp) expressing GAL4 BD alone serve as negative controls.

Examples of different combinations of PP2Cs and RCARs are shown as ten-fold dilution series spottings on selective media (Fig. 9). According to the OD_{600} of each single culture the suspensions were diluted in mQ-H_2O to obtain spots of 10 µl containing 1000, 100 and 10 cells, respectively. Interactions of the candidate proteins allow His-autotrophic yeast proliferation. Non-interacting combinations will not grow. Among the tested combinations, the subgroup of closely related ABI1-like PP2Cs (ABI1, ABI2, HAB1 and HAB2) exhibited constitutive interaction with RCAR3. With the exception of HAB1 the same PP2Cs also constitutively interacted with RCAR7. RCAR11 depended on ABA to stabilize interaction with ABI1-homologs in these assays confirming the results of Park et al. (2009). A similar pattern of RCAR interaction was observed for PP2CA. It constitutively coupled to RCAR3 and RCAR7. With RCAR11 it only interacted in an ABA-dependent fashion. The homologous

PP2Cs HAI2 and HAI3 exhibited ABA-independent interaction with RCAR3 and RCAR7 (Fig. 9). However, they did not interact with RCAR11, irrespective of exogenously supplied ABA. Closely related HAI1 did not show interaction with any of the RCARs tested. ABA-dependent growth on selective media was observed for AHG1 in combination with RCAR3 only. As a negative control At5g27930 PP2C#12 (P#12) was included in the assays presented. Combinations of the PP2Cs with empty BD vectors (emp) served as negative controls. The lack of colony growth in these samples indicates that there is no auto-activation of the reporter. Combinations of non-clade A PP2C P#12 with any of the RCARs did also not proliferate under selective conditions. Alike this, empty AD vector combinations with RCAR3, RCAR7 and RCAR11 were negative on selection plates (data not shown). This also excludes RCAR-BD reporter auto-activation.

The stabilizing effect of ABA on the interaction of PP2Cs and RCARs observed in yeast is reminiscent of the ligand-dependent inactivation of PP2Cs observed *in vitro* (Hao et al., 2011). The binding affinities of different PP2C-RCAR-complexes are different (Szostkiewicz et al., 2010). To analyze the ABA-dependence of co-receptor interaction in detail in yeast, the Y2H liquid culture growth assay (YLGA) based on His-autotrophy was established. Single clones representing combinations of PP2Cs and RCARs were incubated in sterile microtiter cell culture plates containing selective media. Yeast proliferation was determined by intervallic measurement of culture OD_{600} and thereby served as read out for His-reporter activity. Equal amounts of cells ($2*10^4$ cells/well) were used for inoculation. Yeast cells were cultured at 30 °C under constant shaking (200 rpm). OD_{600} values were measured in intervals compared to control media without inoculum. The values reflect yeast proliferation depending on the strength of interaction of the candidate proteins. An aliquot of cells growing in double selection media (-L/-W) served as growth control.

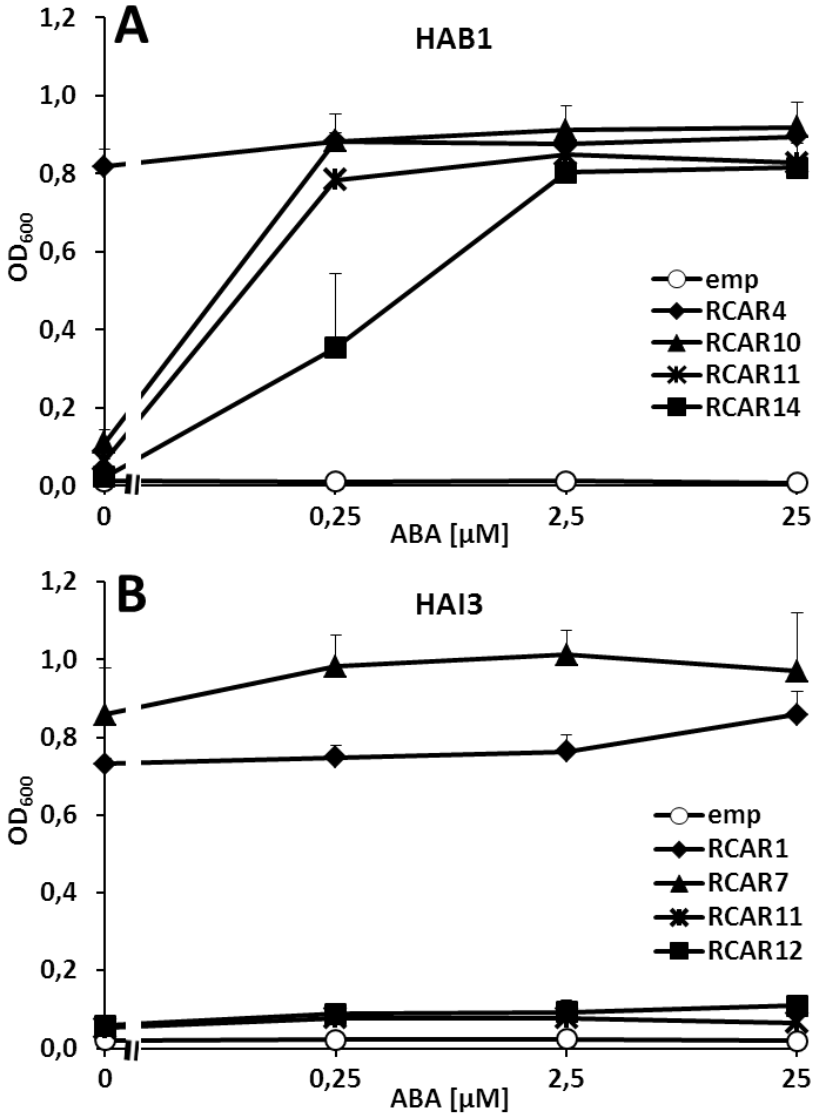


Fig. 10: Y2H interaction of PP2Cs and RCARs. Yeast growth after 64 h incubation in liquid selective medium measured by OD_{600} reflects interaction-dependent His-autotrophy. Triple selective medium (-L/-W/-H) is supplemented with varying concentrations of ABA. Empty vector (emp) combinations serve as negative control. (A) Combinations of HAB1 with RCAR4, RCAR10, RCAR11 and RCAR14 were assayed in liquid culture for ABA-dependent interaction. (B) Combinations of HAI3 with RCAR1, RCAR7, RCAR11 and RCAR12 were assayed in liquid culture for ABA-dependent interaction. Growth ratios of HAB1/RCAR4 and HAI3/RCAR1 combinations in triple selective media relative to -L/-W controls are 0,929 (± 0,028) and 0,838 (± 0,015) for A and B, respectively. Values are means (± SD) of three independent transformant lines.

The proliferation of yeast cells after incubation for 64 h in triple selective media (-L/-W/-H) supplemented with different ABA concentrations is presented in Fig. 10. The interaction of ABI1-like PP2C HAB1 with RCAR4 is not dependent on ABA. Contrastingly, growth of cells harboring HAB1 in combination with RCAR10, RCAR11 and RCAR14 is diminished in the absence of exogenously provided ABA (Fig. 10A). Supplementation of the media with 0,25 µM ABA is sufficient to induce interaction and thus yeast proliferation for all combinations. At concentrations of > 0,25 µM ABA, growth of HAB1 combinations with subfamily III RCAR10 and RCAR11 is similar to RCAR4 constitutive

control. The formation of a HAB1-RCAR14 complex seems to depend on higher ligand concentrations (≥2,5 µM ABA). The proliferation of colonies harboring HAB1 and an empty BD vector (emp) is completely stunted making this an adequate negative control. The same setup was used to test HAI PP2C subfamily member HAI3. The experiments showed constitutive interaction of HAI3 with RCAR1 and RCAR7 (Fig. 10B). In contrast to HAB1, HAI3 did not interact with RCAR11 and RCAR12 from subfamily III, irrespective of the presence of high ABA concentrations. Their OD_{600} values were similar to the empty vector control. Thus, ABA stabilizes complex assembly of only a subset of combinations.

The course of interaction-dependent proliferation of yeast can be visualized in a time-dependent manner. The kinetics and affinities of different co-receptor complexes could be compared. Additionally, the effect of exogenously provided ABA could be investigated. In Fig. 11 the growth of the yeast colonies monitored over time is depicted.

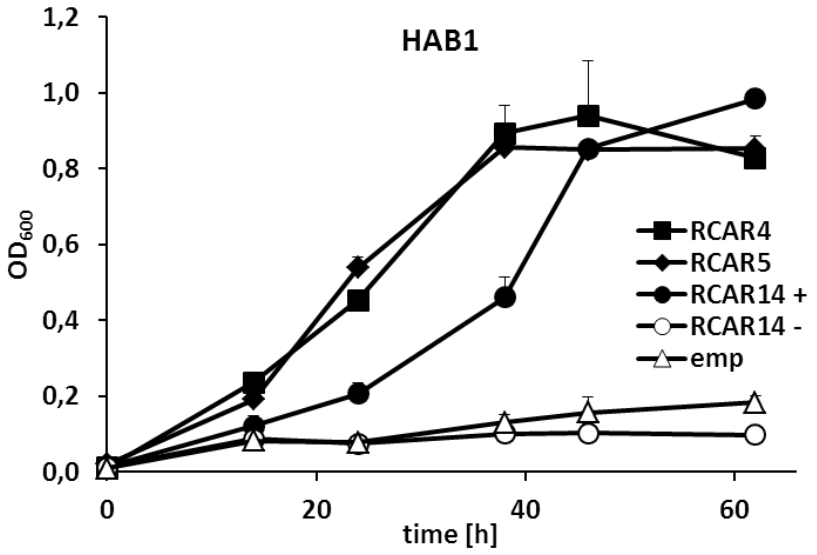


Fig. 11: Proliferation of HAB1-RCAR combinations. The yeast growth in triple selective medium is monitored over time. Values of OD_{600} reflect interaction-dependent His-autotrophy. Combinations of HAB1 with RCAR4, RCAR5, and RCAR14 were monitored in YLGA. Complex stabilization by 25 µM ABA is shown for HAB1-RCAR14 complex (RCAR14 +). An empty pGAD vector (emp) combination serves as negative control. The growth ratio of HAB1/RCAR4 combination in triple selective medium relative to -L/-W controls is 0,937 (± 0,045). Values are means (± SD) of three independent transformant lines.

The proliferation of yeast cells harboring combinations of HAB1 and RCAR4 or RCAR5 in triple selective medium (-L/-W/-H) is similar to the growth in control medium (-L/-W; data not shown). The formation of a complex between HAB1 and RCAR14 was not established in yeast unless exogenous ABA [25 µM] was supplemented in the media (RCAR14 +). In the absence of ABA the proliferation is similar to the empty BD vector control. This illustrates the ligand-dependent formation of the co-

receptor complex in yeast. Comparison of growth curves between constitutively interacting control lines, e.g. HAB1-RCAR4, and HAB1-RCAR14 combination in the presence of 25 µM ABA also revealed that these lines are slightly retarded in their proliferation.

In the AH109 yeast strain used for the present analyses there are also other read-out systems to assess protein-protein interaction. One of these alternative reporters is the GAL4-dependent activation of a β-galactosidase (β-Gal) gene. After cell lysis enzymatic activity on the synthetic substrate o-Nitrophenyl-β-D-galactopyranosid (ONPG) can be quantified fluorometrically to estimate the strength of interaction. To substantiate the interaction data from the His-autotrophy assays, a compilation of PP2C-RCAR combinations was also assayed for β-Gal activity.

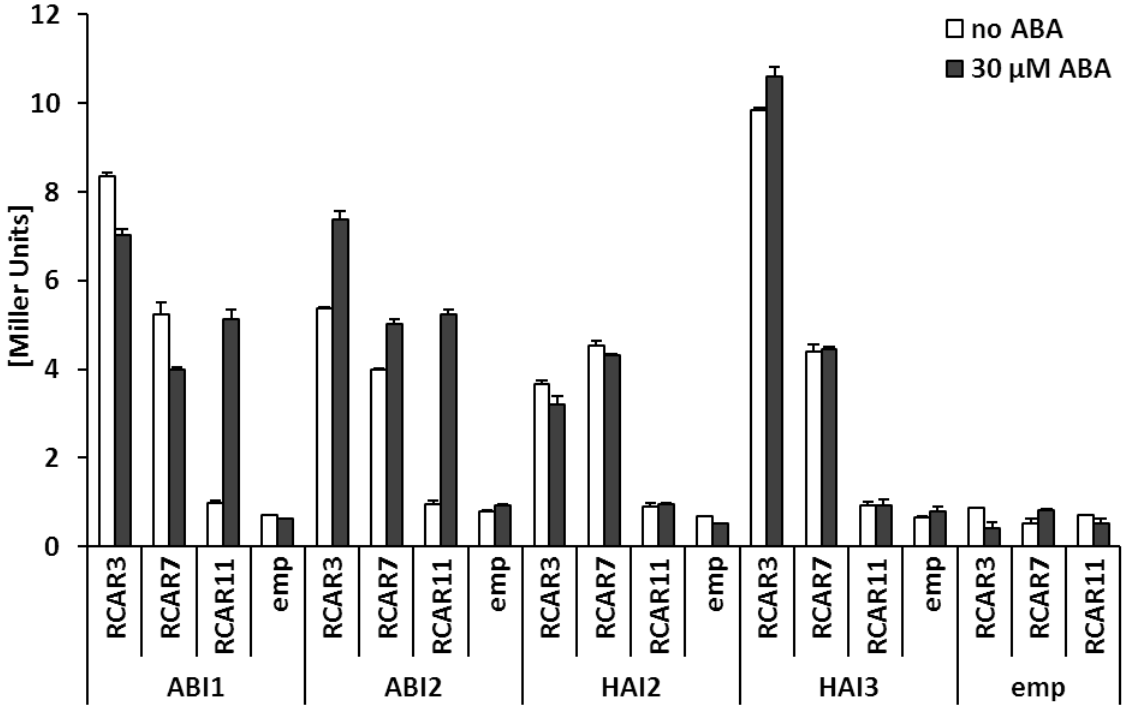


Fig. 12: PP2C-RCAR interaction in β-Gal assay. Exemplary combinations were tested quantitatively for β-Gal activity as a read-out for interaction. Liquid cultured cells were grown without or with ABA [30 µM] in double selection medium (SD -L/-W). Values are means (± SD) of three independent transformant lines and given in Miller Units. Empty vector (emp) combinations serve as negative controls. Values ≤ 1 indicate absence of interaction.

The subfamilies of ABI1-like and HAI-like PP2Cs showed different preferences for RCAR-interaction in the His-autotrophy assays. With ABI1, ABI2 and HAI2, HAI3 two members of the different clade A PP2C subgroups were tested for β-Gal activity. Each of the PP2Cs was assayed for interaction with RCAR3, RCAR7 and RCAR11 representing the RCAR subfamilies I, II and III, respectively (Fig. 12). The quantity of β-Gal activity reflects the strength of protein-protein interaction. ABI1 and ABI2 showed

strong constitutive interaction with RCAR3 at approximately 7 Miller units. The same PP2Cs exhibited constitutive interaction with RCAR7 and ABA-dependent interaction with RCAR11. In the presence of 30 µM ABA the levels of RCAR7 and RCAR11 interaction with ABI1 and ABI2 were comparable. The HAI group PP2Cs HAI2 and HAI3 showed no interaction with RCAR11, confirming the His-autotrophy data (see Fig. 9 and Fig. 10). Their interaction with RCAR7 was similar to that of ABI1 and ABI2, irrespective of ABA. Strikingly, the coupling of HAI3 to RCAR3 with > 10 Miller units was significantly stronger than the values of ABI1, ABI2 (~7 Miller units) or HAI2 interaction (~4 Miller units). The empty vector controls serve as negative controls and showed values < 1 Miller unit.

A variable assembly of co-receptor complexes is confirmed by the data gathered in yeast. The two reporter systems exploited (His-autotrophy and β-Gal activity) produced highly consistent results. Subfamily III RCARs showed ABA-dependent interaction with ABI1-like PP2Cs, confirming the results from Park et al. (2009). The HAI group PP2Cs did not interact with RCARs from subgroup III in yeast. This is in line with the results for HAI PP2Cs obtained by Bhaskara et al. (2012). Consequently, RCARs bind to PP2Cs in variable combinations that partly depend on exogenous ABA for complex formation in yeast. Different binding affinities indicate a combinatorial framework governing signaling outcome. The Y2H analyses performed for all 126 possible combinations of 9 PP2Cs x 16 RCARs are summarized in Fig. 13.

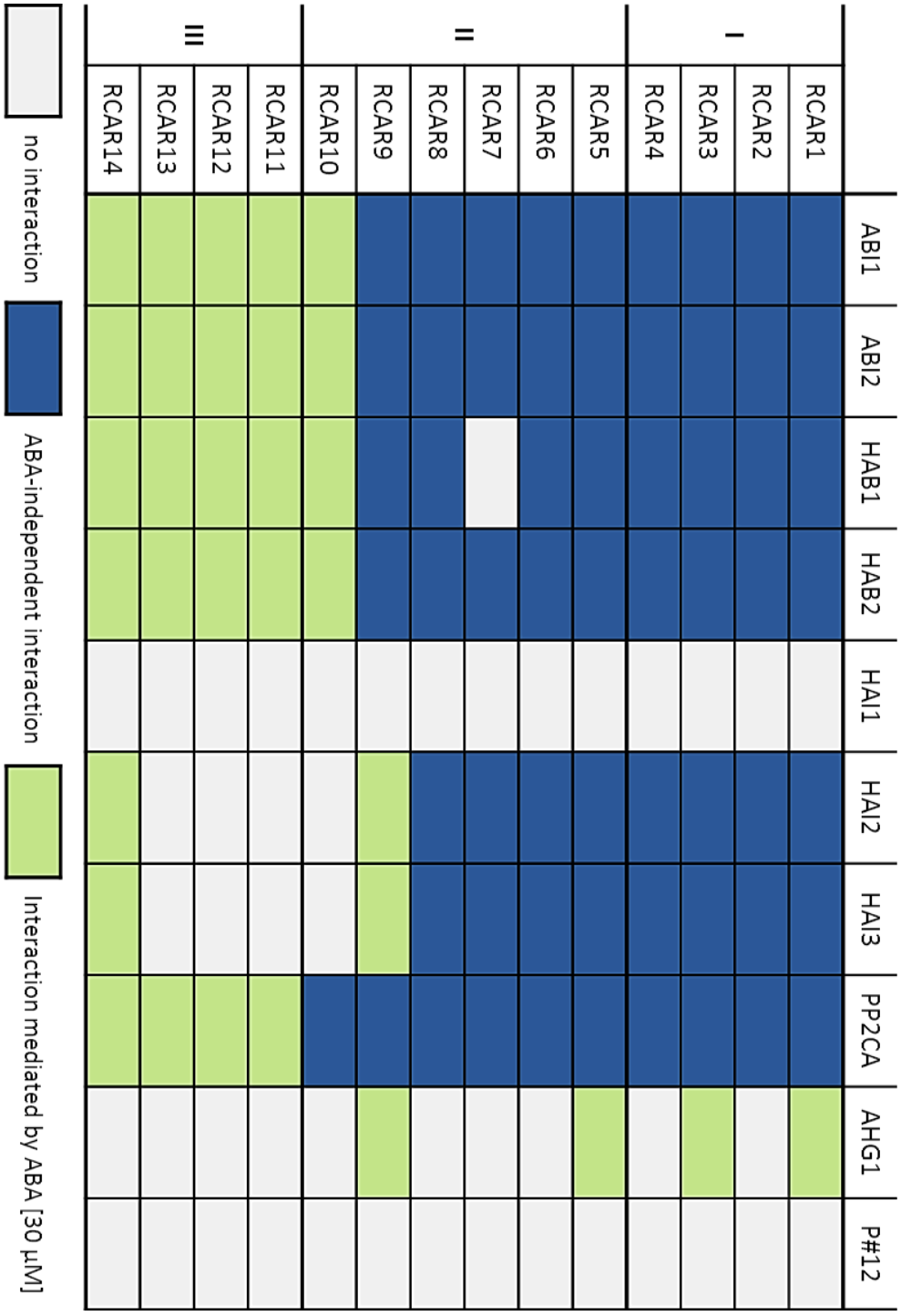


Fig. 13: Summary of PP2C-RCAR interactions. The complement of 126 possible combinations between clade A PP2Cs and RCARs (in AD and BD, respectively) tested for Y2H interaction is presented as a matrix. Clade E PP2C At5g27930 (P#12) is included as a control. Grey, blue and green shading indicate no interaction, ABA-independent interaction and interaction mediated by 30 µM exogenous ABA, respectively.

The multiple combinations of receptors and PP2Cs show a distinct pattern of protein-protein-interaction (Fig. 13). Subgroup I of the RCAR family constitutively interacts with seven out of all nine phosphatases of clade A (ABI1, ABI2, HAB1, HAB2, HAI2, HAI3 and PP2CA). On the other hand, group II and group III RCARs show interaction partly depending on ABA. Most of the clade A PP2Cs tested

show interaction with a subset of RCAR proteins. HAI1 is the only phosphatase, which does not show any interaction in the assay systems used, neither in the presence or absence of the ligand. In the case of AHG1 solely a certain subset of RCARs (RCAR1, RCAR3, RCAR5 and RCAR9) can interact in the presence of exogenous ABA. Such a ligand-dependent stabilization is seen for several RCARs. For example, the proteins out of subgroup III did not stably interact with any of the tested phosphatases in yeast. Only in the presence of the ligand the binding was stabilized, which is conform to previous data on HAB1 and its interaction with RCAR proteins gathered by Park et al. (2009). This versatile pattern of PP2C and RCAR coupling in yeast indicates preferential interaction. The single combinations feature different affinities and this constitutes the basis of a complex network of ABA perception relays.

2.1.2 PP2C-RCAR interference *in vivo*

The heterologous interaction studies presented here and a number of *in vitro* analyses (Antoni et al., 2012; Melcher et al., 2010a; Szostkiewicz et al., 2010) showed a specific pattern of RCAR docking resulting in PP2C inactivation. To confirm the results gathered in yeast, a transient gene expression system in protoplasts was used for *in vivo* studies (Sheen, 2001). PP2C and RCAR components were ectopically expressed in Arabidopsis mesophyll protoplasts and surveyed for the induction of an ABA-responsive reporter (Yoo et al., 2007). The reporter construct consists of the promoter region of the Responsive to Desiccation 29B (RD29B) gene (Yamaguchi-Shinozaki and Shinozaki, 1993) driving the expression of the firefly *Photinus pyralis* luciferase (LUC) enzyme. This pRD29B::LUC reporter is specifically and strongly induced by ABA and therefore used to monitor ABA-dependent gene regulation and signal transduction (Christmann et al., 2005; Msanne et al., 2011; Nakashima et al., 2006). A constitutively expressed bacterial β-glucoronidase (β-GUS) reporter gene served as internal control for transfection and as a standard for normalization (for details see 4.2.11).

For the ectopic expression of PP2C and RCAR effector proteins, the CDS fragments of the respective genes were transferred from the present Y2H constructs into a plant expression construct. This vector is based on a pBluescript II SK (Alting-Mees et al., 1992) derivative generated at the institute (Hoffmann, 2001). Expression is driven by a highly active Cauliflower Mosaic Virus (CaMV)-derived 35S promoter (Benfey and Chua, 1990; Odell et al., 1985) and aborted by the Nopalin synthethase (Nos) terminator sequence. A new vector (#4065 in the institute´s strain collection) with a multiple cloning site (MCS) compatible to the Y2H vectors used was generated (see 4.2.12 for details). This allowed immediate transfer and testing of all nine PP2Cs and all 14 RCARs *in vivo*.

In the protoplast system used, the ABA response is quantified as pRD29B::LUC activity relative to the co-transfected constitutive 35S GUS internal standard. This ABA-dependent reporter activation was shown to be blocked or enhanced by co-expression of clade A PP2Cs or RCARs, respectively (Fujii et al., 2009; Ma et al., 2009). To compare the effectiveness of different PP2Cs and different RCARs in regulating ABA-dependent signal transduction, a series of protoplast experiments were performed. For each of the RCAR subfamilies one member was exemplarily co-expressed and the effect of different PP2Cs was assessed.

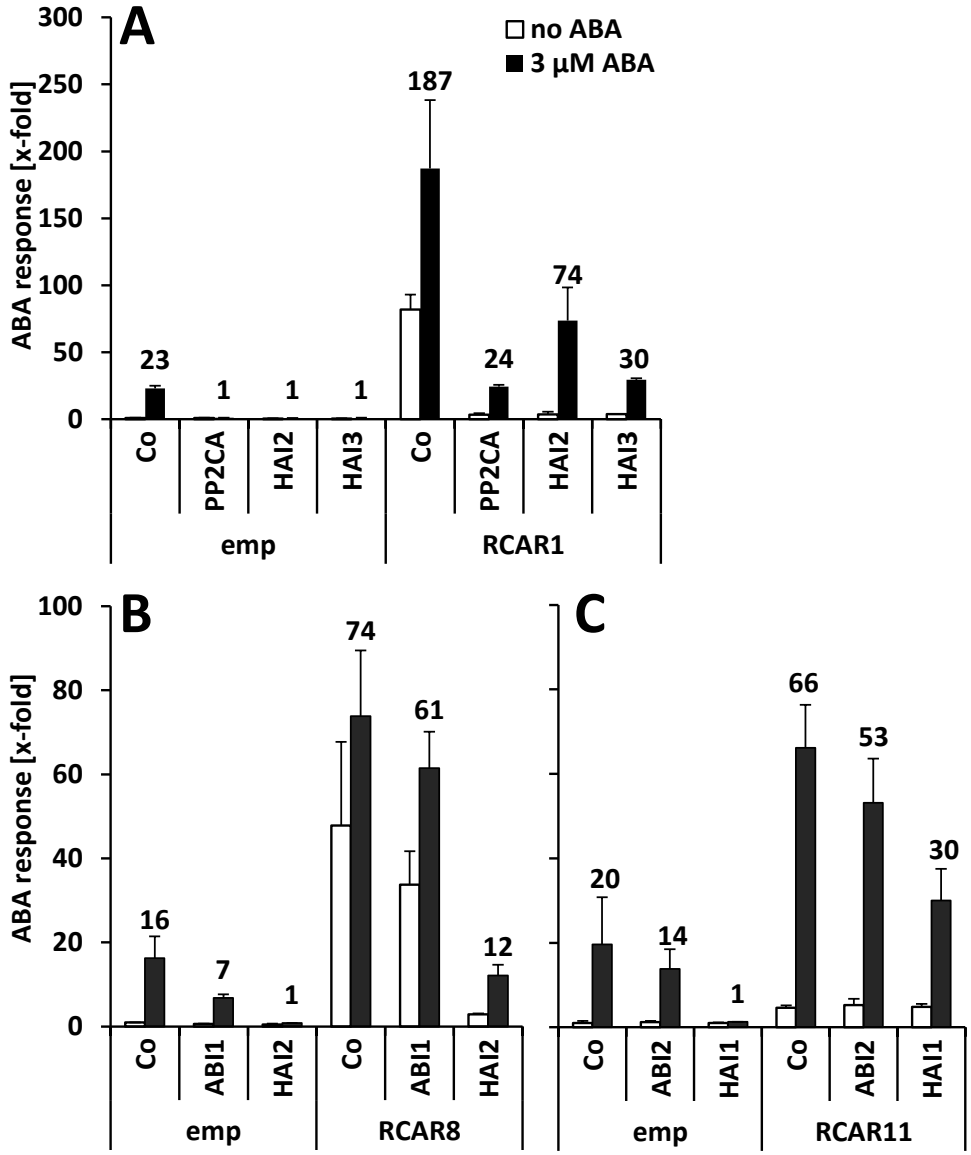


Fig. 14 : ABA signaling in protoplasts controlled by PP2Cs and RCARs. Different PP2Cs were co-expressed with the empty vector (emp) or RCAR1 (A), RCAR8 (B) or RCAR11 (C). Signaling activation is presented as x-fold induction of the ABA-response compared to untreated empty vector control (Co). Induction values of the samples supplied with exogenous ABA are provided. Samples were incubated for 20 h without or with 3 µM ABA. For each construct 1 µg of RCAR and PP2C effector plasmid DNA was transfected. Mean GUS activities are 4,472 (± 0,784), 1,753 (±0,261) and 0,431 (±0,071) RFU/sec for experiments A, B and C, respectively.

There is a clear difference in the extent of repression or activation of ABA-dependent gene regulation between different PP2Cs and RCARs, respectively (Fig. 14). Hao et al. (2011) reported ligand-independent inactivation of PP2Cs by subfamilies I and II RCARs *in vitro*. This is consistent with protoplast experiments shown, where the response is strongly induced in the absence of the ligand ABA by an overexpression of RCAR1 (Fig. 14A) and RCAR8 (Fig. 14B), but weakly by RCAR11 (Fig. 14C). In general, the empty vector (emp) controls (Co) of all three experiments show an approximately 20-fold increase of the signal in the presence of 3 µM ABA in Col wt protoplasts. This effect is completely abolished by the co-expression of PP2CA, HAI1, HAI2 or HAI3. Co-expression of ABI1 and ABI2 partly reduces ABA-responsive reporter activation. Without exogenously supplied ligand, ectopic levels of RCAR1 and RCAR8 increase ABA response around 80- and 50-fold, respectively. For RCAR11 an ABA-independent induction is not observed. In the presence of exogenous ABA the RCAR-mediated signaling activation is augmented. Co-expression of RCAR1, RCAR8 and RCAR11 in ABA treated samples amplifies the ABA response up to 187-, 74- and 66-fold induction, respectively.

The effect of different PP2Cs on RCAR-elicited signaling induction is variable. PP2CA and HAI3 efficiently reduced RCAR1-mediated response in the presence of exogenous ABA down to levels similar to control samples (Fig. 14A). Co-expression of HAI2 only partly compensated the inductive effect of RCAR1. In contrast to this, HAI2 efficiently blocked RCAR8-mediated up-regulation of reporter activity (Fig. 14B). ABA response values in the presence of the ligand were reduced from 74- to 12-fold. ABI1 weakly interfered with RCAR8 induction. In RCAR11-dependent signaling without exogenous ABA there was no significant effect of the PP2Cs tested ABI2 and HAI1. If the samples were treated with 3 µM ABA, HAI1 was more effective than ABI2 in blocking the signal transduction. HAI1 co-expression caused approximately a 50 % reduction of the signal. The general function of RCAR proteins as positive regulators of ABA signaling is confirmed here (Fujii et al., 2009; Klingler et al., 2010; Umezawa et al., 2010). Furthermore, ectopic expression of clade A PP2Cs differentially blocks ABA-dependent and RCAR-mediated signal transduction. As there are 14 proteins within the RCAR family, the effect of other family members on PP2C activity was to be investigated. According to the heterologous studies in yeast, HAI3 features partly ABA-dependent interactions with a subset of RCARs (compare Fig. 13). Hence, a selection of RCARs from the three different subgroups in combination with HAI3 PP2C was analyzed regarding their combinatorial effect on ABA-dependent gene regulation (Fig. 15).

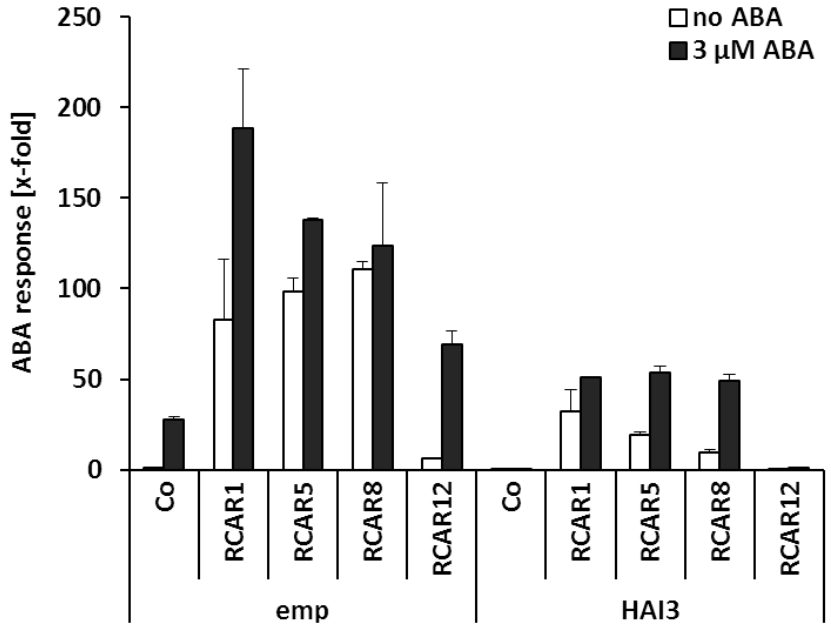


Fig. 15: ABA signaling in protoplasts controlled by RCARs and HAI3. Different RCARs were co-expressed with the empty vector (emp) or with HAI3. Signaling activation is presented as x-fold induction of the ABA response compared to untreated empty vector control (Co). Samples were incubated for 20 h without or with 3 µM ABA. For each effector construct 1,5 µg of plasmid DNA was transfected. The mean GUS activity is 0,799 (± 0,105) RFU/sec for these samples.

The extent of ABA signaling activation clearly varies between different RCAR proteins (Fig. 15). Expression of RCAR1, RCAR5, or RCAR8 strongly induced reporter activity without exogenous ABA. This constitutive activation was significantly weaker for RCAR12. The co-expression of HAI3 completely abolished ABA-dependent reporter activation in the control samples (Co). HAI3 function also significantly reduced RCAR-induced ABA response. In the case of RCAR12, the RCAR-mediated increase of reporter activity was fully abolished by HAI3.

The results depicted in Fig. 14 and Fig. 15 vastly agree with the experiments in yeast (Fig. 13). RCARs of subgroup I and most RCARs of subgroup II interacted constitutively with many of the PP2Cs in the Y2H assays. The same RCARs significantly activated ABA signaling in protoplasts without the application of exogenous ABA. RCAR1-mediated signaling was further enhanced by supplying the ligand. In the case of RCAR5 and RCAR8 the application of exogenous ABA did not drastically increase the autonomous induction. These *in vivo* results confirm a complex combinatorial interaction of PP2Cs and RCARs and the subsequent regulatory outcome in ABA-dependent signaling. The get more detailed information about the functional relation of the proteins in the regulation of ABA signaling, titration experiments of RCARs and PP2Cs were performed.

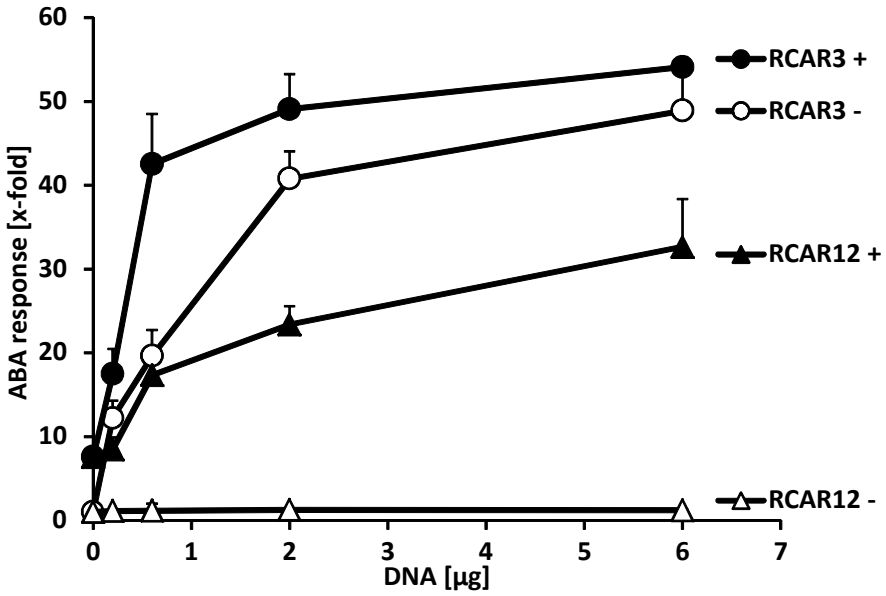


Fig. 16: RCAR-dependent activation of ABA signaling. Different amounts of RCAR3 (circles) and RCAR12 (triangles) effector plasmids were transfected in Arabidopsis protoplasts. Signaling activation is presented as x-fold induction of the ABA response compared to untreated empty vector control (no RCAR and no ABA). Samples were incubated for 20 h without (open symbols) or with 5 µM ABA (black symbols). Mean GUS activities of these samples are 0,163 (± 0,079) RFU/sec.

Subfamily I member RCAR3 features ABA-independent interaction in yeast and ABA-independent induction of ABA response in protoplasts. RCAR12 belongs to subfamily III of the RCARs and is dependent on the ligand to establish Y2H interaction and signaling induction in protoplasts (compare Fig. 13 and Fig. 15). The effects on ABA-dependent gene expression mediated by RCAR3 and RCAR12 are compared in Fig. 16. With increasing amounts of RCAR3 in the cells, the ABA-responsive promoter was highly activated. In general, this effect was comparable in the samples with or without exogenous ABA. Only at low RCAR levels ABA leads to a doubling of the response compared to non-induced samples (RCAR3 -). Contrastingly, RCAR12 alone was not sufficient to induce reporter gene activity. In the case of RCAR12 exogenous ABA was necessary to generate a strong increase in ABA signaling compared to non-treated controls (RCAR12 -). This is in line with the data collected for subfamily I and III RCARs in yeast and protoplasts. RCARs differentially regulate ABA signaling outcome *in vivo*.

Previous reports show that clade A PP2Cs exert distinct functions *in planta* (Bhaskara et al., 2012) and are differentially regulated *in vitro* and *in vivo* (Antoni et al., 2012). Based on the interaction analyses from yeast, the protoplast system allows further *in vivo* investigation of the functional consequences of distinct PP2C-RCAR interactions.

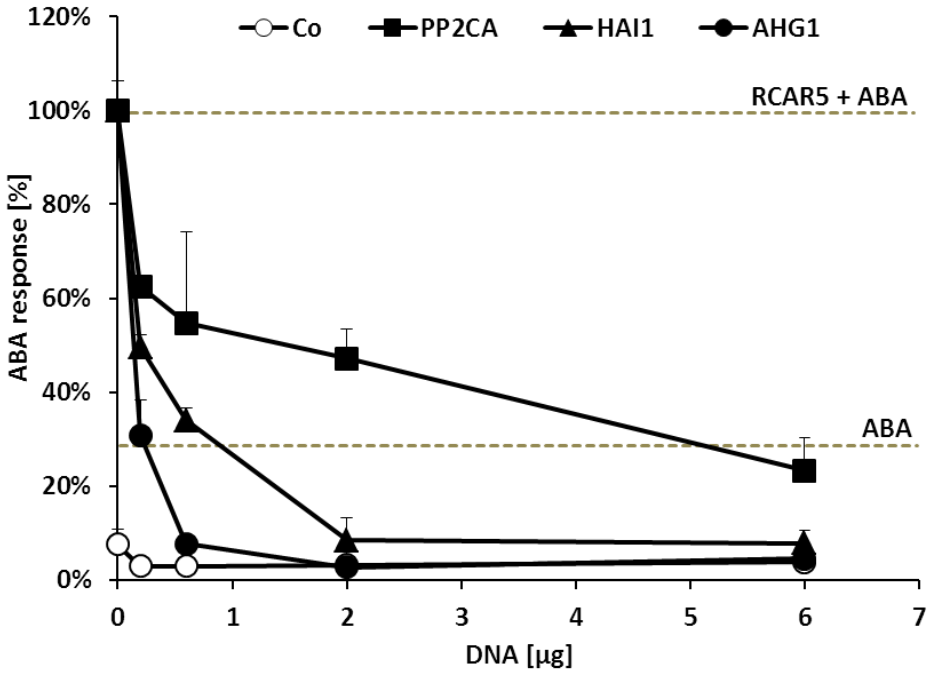


Fig. 17: PP2C-dependent inactivation of ABA signaling in protoplasts. RCAR5-induced reporter activity in the presence of 5 µM ABA is set as 100% ABA response. Graphs illustrate the dose-dependent repression of signaling in the presence of ABA by co-expression of PP2CA (squares), HAI1 (triangles) or AHG1 (filled circles). The AHG1-mediated inhibition of RCAR5-induced reporter activity in the absence of ABA is shown as control (Co, open circles). Dashed lines indicate ABA response levels of RCAR5 with ABA (RCAR5+ABA) and ABA alone (ABA). Mean GUS activities of these samples are 0,116 (± 0,028) RFU/sec.

To confirm a selective inhibition of PP2Cs by a subset of RCARs, co-expression experiments were performed in protoplasts. Fig. 17 shows the PP2C-specific inactivation of RCAR5-elicited signaling. The reporter induction caused by transfection of 600 ng RCAR5 effector DNA in the presence of 5 µM is set to 100 % (RCAR5 + ABA). The effects of different amounts of PP2CA, HAI1 and AHG1 are shown as relative changes in ABA response. AHG1 exhibited the strongest inhibition of ABA response among the PP2Cs tested. It depleted RCAR5-mediated activation down to control levels without ABA (Co) already at concentrations below 1 µg of transfected plasmid DNA. HAI1 concentrations had to be higher than 2 µg of effector plasmid per transfection to efficiently counteract RCAR5-dependent signaling induction. PP2CA co-expression did not cause ABA response repression in this experiment even at high concentrations above 6 µg of transfected vector. For comparison, a dashed line (ABA) indicates the level RCAR5-independent induction of the pRD29B::LUC reporter by 5 µM ABA.

Taken together, the data retrieved from heterologous Y2H analyses and the *in vivo* protoplast studies are highly consistent. PP2C subgroups and RCAR subfamilies exert preferential binding to one another. The coupling and functional regulation is extensively affected by ABA. This ligand-dependent combinatorial assembly of the co-receptor complex constitutes the major molecular relay

in ABA signal transduction. Another globally important part of ABA-related physiological plant stress response encompasses transcriptional reprogramming.

2.2 Transcriptional regulation of ABA signaling components

2.2.1 Expression of PP2Cs

To better understand the concurrence of the many possible receptor-PP2C complexes *in vivo,* expression patterns of PP2Cs and RCARs were analyzed *in silico*. Co-expression in a timely and spatial manner is the basis for direct functional regulation of different receptor complexes. A plethora of transcriptomic datasets and a number of analysis tools is available at the Botany Array Resource (BAR) (http://bar.utoronto.ca/welcome.htm). To study the transcriptional regulation of the genes encoding the core ABA signaling components, the eNorthern tool was exploited (Toufighi et al., 2005). This web-based application allows the graphical presentation of publicly available microarray datasets. Expression levels of multiple genes of interest can be compared between different developmental stages, single tissues or during stress response.

Based on its function in environmental sensing and stress signaling, ABA also serves a major regulator of plant development (Finkelstein et al., 2002; Karssen et al., 1983; Razem et al., 2006; Stone et al., 2006). The hormone causes grave alterations in the transcriptome (Barrero et al., 2005; Hoth et al., 2002; Kreps et al., 2002). By this it is also linked to the processes regulating plant cell and life cycle (Chaves et al., 2009; Pourtau et al., 2004; Rock and Zeevaart, 1991; Wang et al., 1996). The function of clade A PP2Cs might be reflected by their expression patterns throughout Arabidopsis plant life, which has been classified in defined developmental stages by Boyes et al. (2001).

stage	Control	1.00	1.02	1.04	1.06	1.06	1.09	1.09	1.12	6.10
tissue		Seedling	Leaf 1+2	Leaf 4	Root	Leaf 6	Leaf 9	Rosette	Rosette	Leaf Sen
AHG1	0,006	0,57	0,50	0,42	2,03	0,43	0,44	0,63	0,33	0,89
PP2CA	0,124	0,45	0,83	0,54	1,03	1,18	0,55	0,83	1,05	3,36
HAI1	0,043	0,34	0,41	0,37	0,51	0,51	0,35	0,63	0,63	4,51
HAI2	0,009	0,38	0,23	0,25	0,27	0,21	0,19	0,48	0,72	12,78
HAI3	0,005	1,11	1,60	1,64	0,43	0,58	0,70	0,91	0,77	1,08
HAB2	0,036	0,82	0,81	0,78	0,90	1,05	0,79	0,91	1,06	2,78
HAB1	0,101	0,83	0,57	0,68	0,81	1,01	0,54	0,72	1,13	3,97
ABI1	0,095	0,76	0,50	0,57	1,03	0,67	0,50	0,82	1,09	4,79
ABI2	0,017	0,92	0,41	0,52	0,88	0,80	0,46	0,90	1,23	5,80

change in expression [x-fold]

0,10	0,33	0,66	1,00	3,33	6,66	10,00

<10x down-regulated up-regulated **>10x**

Fig. 18: Expression of PP2Cs throughout plant development. The expression of nine clade A PP2Cs is shown at different developmental time points relative to the developmental baseline expression median of the respective gene. Transcript abundance relative to ubiquitously expressed reference gene UBQ10 is indicated by the ratio given in the control column. Intensities of blue and orange indicate transcriptional down- and up-regulation, respectively. Developmental stages and tissues are indicated on top (Boyes et al., 2001; Kilian et al., 2007; Schmid et al., 2005). Grey shading of stages reflects plant age from seedling to senescent plant (see text for details).

Developmental changes of expression are seen for all clade A PP2Cs investigated (Fig. 18). Interestingly, the transcript level abundance compared to ubiquitously expressed At4g05320 UBQ10 reference gene is low in early stages and young and developing tissues. This early vegetative time span comprises samples starting from the complete opening to the cotyledons (stage 1.0) up to the establishment of the full rosette (stages 1.x – where x is the number of fully differentiated leaves). In an unstressed, pre-senescent state, particularly AHG1 and the HAI group PP2Cs are expressed at minimal levels of 0,3 up to 1 % relative to the UBQ10 reference gene. PP2CA and the ABI1-like PP2Cs are detected at slightly higher amounts in these pre-senescent vegetative tissues. The maxima for most of the PP2C genes monitored are seen in senescent leaves. The corresponding stage 6.10 is defined as the time point, at which one tenth of the flowers to be produced are open. And samples are taken from the oldest rosette leaves. Only AHG1 and HAI3 do not peak in these late stages. Their transcripts are more abundant in roots and young rosette leaves (stage 1.4), respectively. A relatively strong up-regulation in senescing tissues is seen for HAI2, which is more than 30-fold induced compared to the levels in unstressed seedlings of stage 1.0. Overall, low expression levels at the pre-senescent vegetative phases and a subsequent induction in the course of senescence are characteristic for all clade A PP2Cs. This implies analogous transcriptional regulation for this group of phosphatases.

The later stages in the Arabidopsis life cycle not only include senescence of older parts but also the seed maturation following successful reproduction. As clade A PP2Cs are known to be major regulators of germination control, their expression in the course of seed development and germination was studied. The transcriptomic data visualized in Fig. 19 come from the AtGenExpress datasets available at the BAR (Schmid et al., 2005; Toufighi et al., 2005). This array was designed to specifically track the transcriptional control underlying seed maturation and germination traits.

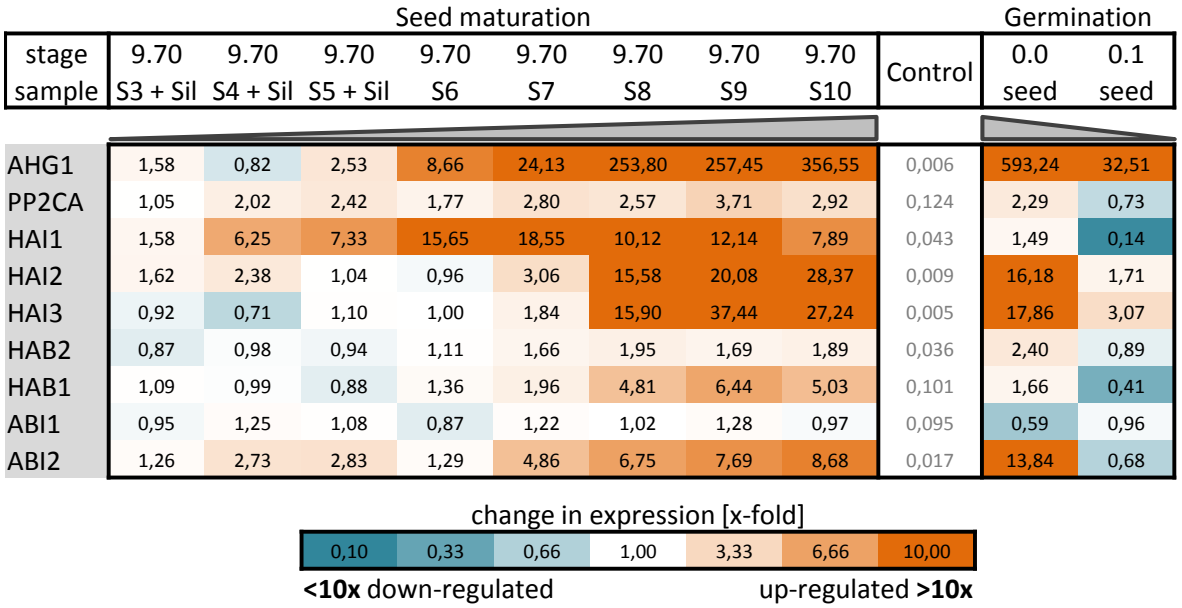


Fig. 19: Expression of PP2C genes in seed maturation and germination. PP2C expression is shown at different time points relative to the developmental baseline expression median of the respective gene. Transcript abundance relative to ubiquitously expressed reference gene UBQ10 is indicated by the ratio given in the control column. Intensities of blue and orange indicate transcriptional down- and up-regulation, respectively. Developmental stages and samples are indicated on top (Boyes et al., 2001; Kilian et al., 2007; Schmid et al., 2005). The grey slopes symbolize increasing seed maturity and decreasing seed dormancy status, respectively.

The transition from vegetative to reproductive growth is a vitally important step in plant development, especially for herbaceous plants. Seed production after fertilization is a key-step in the plant life cycle and it is tightly regulated. ABA integrates environmental stimuli and downstream signaling is regulated by clade A PP2Cs. The regulation of PP2C transcript levels is tracked across the different phases in seed maturation and germination in Fig. 19. The samples representing the early stages in seed development include silique material (samples S3 - S5). Later stages refer to embryonal maturation (samples S6 - S10). Stages 0.0 and 0.1 represent dry and imbibed seeds, respectively. The expression levels of AHG1 and the HAI PP2Cs are strongly induced in ripening siliques and seeds. HAI transcripts are more than 20-fold up-regulated. AHG1 levels peak in the mature seed, where it is expressed nearly 600-fold. This indicates an approximately 3,5-fold more

abundant transcript level of AHG1 over the constitutively expressed reference gene UBQ10. This regulation coincides with its dominant role in germination traits (Nishimura et al., 2007). Minor induction in the course of seed maturation is seen for PP2CA, HAB1 and HAB2. On the other hand ABI1 appears not to be significantly regulated in this process.

In the dry seeds (stage 0.0) and directly after imbibition (stage 0.1) the levels of the protein phosphatase transcripts drop significantly. PP2CA, HAI1 and the ABI1-like PP2Cs are down-regulated below control levels. Only the expression of AHG1 is retained at high levels (32-fold compared to baseline control) even beyond the earliest seedling stages, up to the complete opening of the cotyledons (stage 1.0). Generally the expression of the clade A PP2Cs decreases throughout germination and is very low compared to UBQ10 reference gene in seedlings (compare Fig. 18). Hence, the importance of the PP2Cs in seed formation and germination is reflected by their expression pattern.

To investigate the transcriptional regulation of the PP2Cs during stress perception and response, the variation of the respective transcript levels was analyzed based on the array data available from the AtGenExpress database. The samples used for the analyses were prepared from 18 day old Col wt seedlings, grown under hydroponic culture conditions (Kilian et al., 2007). The values inferred from the BAR eNorthern tool indicate the average of three independent biological replicates. The relative alterations of clade A PP2C expression under osmotic, salt and drought stress regimes were monitored (Fig. 20).

A

	Control	0,5	1	3	6	12	24	[h]
AHG1	0,001	2,80	0,79	0,65	1,58	9,16	56,13	OSMOTIC STRESS
PP2CA	0,119	0,76	5,07	6,67	7,70	10,29	7,64	
HAI1	0,010	1,16	2,15	28,66	46,23	56,81	58,38	
HAI2	0,002	1,91	16,48	80,38	63,21	146,39	186,87	
HAI3	0,005	1,14	0,83	0,85	1,13	14,15	26,09	
HAB2	0,022	0,91	1,14	2,01	2,27	2,61	2,82	
HAB1	0,081	0,96	2,01	7,62	12,88	7,18	6,45	
ABI1	0,027	1,22	4,70	12,21	9,98	9,58	10,68	
ABI2	0,009	1,26	3,29	13,95	14,82	15,88	12,49	

B

AHG1	0,001	1,13	0,98	0,59	0,61	2,89	19,42	SALT STRESS
PP2CA	0,119	1,02	3,06	7,18	6,46	6,02	4,51	
HAI1	0,010	1,38	1,47	18,57	9,44	13,50	19,65	
HAI2	0,002	1,75	11,14	50,49	25,79	57,74	60,10	
HAI3	0,005	1,01	0,68	1,30	1,42	1,15	2,40	
HAB2	0,022	1,19	1,08	2,11	1,97	1,51	1,65	
HAB1	0,081	1,01	1,26	6,20	4,94	3,14	2,74	
ABI1	0,027	1,68	2,01	8,75	5,50	5,76	5,37	
ABI2	0,009	1,56	2,29	13,07	8,22	6,30	4,95	

C

AHG1	0,001	1,10	2,92	0,42	1,14	0,47	1,06	DROUGHT STRESS
PP2CA	0,119	2,83	7,84	1,62	1,16	1,01	1,38	
HAI1	0,010	5,40	5,23	1,29	1,24	0,94	1,21	
HAI2	0,002	8,21	33,85	2,55	1,25	1,49	3,01	
HAI3	0,005	0,74	0,97	0,65	0,83	0,70	1,08	
HAB2	0,022	1,41	1,43	1,00	1,03	0,89	0,92	
HAB1	0,081	1,74	1,90	1,17	1,49	1,00	1,11	
ABI1	0,027	8,34	8,00	2,12	1,91	1,10	1,34	
ABI2	0,009	3,56	4,30	2,06	1,29	0,95	1,10	

change in expression [x-fold]

0,10	0,33	0,66	1,00	3,33	6,66	10,00

<10x down-regulated up-regulated **>10x**

Fig. 20: Expression of PP2C genes under stress conditions. PP2C expression relative to untreated control is shown at different time points (0-24 h) after (A) osmotic, (B) salt or (C) drought stress treatment. Transcript abundance relative to ubiquitously expressed reference gene UBQ10 is indicated by the ratio given in the control column. Intensities of blue and orange indicate down- and up-regulation, respectively.

Prolonged exposure of Arabidopsis to osmotic, salt or drought stress causes distinct changes in PP2C transcript abundance (Fig. 20). Hyperosmolarity due to 400 mM mannitol supplemented in the growth medium triggers generally increased PP2C expression for all but HAB2 (Fig. 20A). HAB2 transcript levels hardly increase upon osmotic stress. HAB1, PP2CA, ABI1 and ABI2 are approximately 10-fold up-regulated. AHG1 and the HAI phosphatases are markedly induced with approximately 50-

fold expression compared to the developmental baseline control. HAI2 transcript accumulates 186-fold compared to the control after 24 h stress treatment. AHG1 and HAI3 levels increase after prolonged stress exposure for 12 - 24 h. The transcripts of the other osmotic stress-responsive PP2Cs accumulate rapidly after 1 - 3 h of treatment.

The induction of the phosphatases at the transcriptional level under salt stress treatment with 150 mM NaCl in the growth medium is not as pronounced as under osmotic stress (Fig. 20B). In particular, HAI1 and HAI2 react on the hypersaline stimulus with a strong sustained up-regulation. This is consistent with the reports that had identified the HAI clade A PP2Cs based on their strong transcriptional induction by salt or drought stress (Fujita et al., 2009). HAB1, PP2CA, ABI1 and ABI2 exhibit a transient induction (6-, 7-, 8-, and 13-fold, respectively) at 3 h stress treatment. After this initial increase, transcription drops again. Osmotic stress marginally alters HAB2 and HAI3 transcription. AHG1 features a transient decrease after 1 - 3 h of stress treatment before high transcript levels accumulate after a 24 h treatment. A similar expression pattern of AHG1 is observed under osmotic stress.

Strikingly, the exposure of the seedlings to drought stress does not stably raise PP2C transcript levels (Fig. 20C). Only HAI1, PP2CA, ABI1 and HAI2 show a more than 5-fold induction after 1 h of treatment. The other PP2Cs are not significantly affected by the drought stress treatment. These comparably weak and transient changes are mainly due to the limitations regarding experimental drought stress simulation. The plant material was transiently deprived of water for 15 min before being put back to normal growth conditions (Kilian et al., 2007).

Nevertheless, there is a clear, expeditious and characteristic transcriptional response of the clade A phosphatases to all the three different types of stress imposed.

Stress response and developmental control are tightly interconnected via the complex network of hormonal modulation. Both are mediated through and regulated by a set of phytohormones. For example, developmental cues are mediated by gibberellins (Zentella et al., 2007), while abiotic stress stimuli are associated with the accumulation of ABA (Chinnusamy et al., 2004; Zhu, 2002). The effect of six major phytohormones, namely ethylene (ACC), cytokinin (Zeatin), methyl-jasmonic acid (MeJA), auxin (IAA), ABA and gibberellin (GA3) on the expression of clade A PP2Cs is shown in Fig. 21.

treatment time [h]	control	ACC [10 µM] 0,5	1	3	control	Zeatin [1 µM] 0,5	1	3	control	MeJA [10 µM] 0,5	1	3
AHG1	0,004	1,07	3,27	1,05	0,004	0,41	1,83	0,71	0,004	0,91	1,09	0,55
PP2CA	0,040	1,18	1,13	0,86	0,040	0,87	1,10	1,05	0,040	1,06	1,01	1,08
HAI1	0,005	1,91	1,21	0,92	0,005	1,39	0,92	1,28	0,005	1,35	0,95	1,44
HAI2	0,003	1,57	1,80	1,26	0,003	0,78	1,16	1,45	0,003	0,58	0,78	1,00
HAI3	0,002	1,32	3,56	0,53	0,002	1,03	2,65	0,43	0,002	0,48	1,16	0,43
HAB2	0,015	0,84	0,87	0,91	0,015	0,74	0,75	0,86	0,015	1,08	1,46	1,19
HAB1	0,037	0,95	1,08	0,86	0,037	0,94	1,04	0,68	0,037	1,02	0,93	0,81
ABI1	0,034	1,22	2,31	1,87	0,034	0,69	0,59	0,75	0,034	1,64	2,38	1,91
ABI2	0,003	0,51	1,93	1,23	0,003	1,90	4,33	1,06	0,003	1,03	1,62	0,98

treatment time [h]	control	IAA [1µM] 0,5	1	3	control	ABA [10 µM] 0,5	1	3	control	GA3 [1µM] 0,5	1	3
AHG1	0,004	0,61	0,79	1,22	0,004	2,34	1,86	0,93	0,004	0,82	0,61	1,62
PP2CA	0,040	0,83	1,17	1,07	0,040	4,47	7,57	11,66	0,040	0,89	1,13	1,34
HAI1	0,005	1,18	0,59	1,49	0,005	4,01	12,08	52,19	0,005	2,00	0,80	0,60
HAI2	0,003	0,74	0,86	0,62	0,003	1,98	6,35	42,10	0,003	0,91	1,08	1,62
HAI3	0,002	1,02	0,92	0,65	0,002	0,99	9,72	57,18	0,002	1,12	2,06	1,99
HAB2	0,015	0,81	0,84	1,14	0,015	1,23	2,03	4,41	0,015	0,75	0,80	0,89
HAB1	0,037	0,98	1,06	1,10	0,037	2,17	4,49	10,66	0,037	0,98	1,08	0,92
ABI1	0,034	1,23	1,18	1,89	0,034	5,47	11,33	14,02	0,034	1,02	0,86	0,80
ABI2	0,003	0,91	3,59	2,58	0,003	6,28	48,25	64,58	0,003	1,29	2,37	0,78

change in expression [x-fold]

0,10	0,33	0,66	1,00	3,33	6,66	10,00

<10x down-regulated up-regulated **>10x**

Fig. 21: Expression of PP2C genes after hormone treatment. Expression levels of clade A PP2Cs relative to untreated controls are shown at different time points (0-3 h) after ACC, zeatin, MeJA, IAA, ABA and GA3 treatment. Transcript abundance relative to ubiquitously expressed reference gene UBQ10 is indicated by the ratio given in the control column. Intensities of blue and orange indicate down- and up-regulation, respectively.

The AtGenExpress database also includes datasets on the effect of hormone treatment on Arabidopsis seedlings (Schmid et al., 2005). Transcriptional response over time (0 - 3 h) was analyzed for the clade A PP2Cs (Fig. 21). Among the hormones tested exclusively ABA markedly activates the expression of the phosphatases. Already after a 3 h treatment with 10 µM ABA the HAI PP2Cs and ABI2 are more than 40-fold induced. ABI2 shows the strongest response with approximately 50-fold induction after 1 h. HAB1, PP2CA and ABI1 are still more than 10-fold up-regulated after 3 h. HAB2 levels increases 4-fold. AHG1 transcript abundance is not significantly affected by exogenous ABA.

Other phytohormones like MeJA and GA3 only marginally influence the transcription of the genes investigated. ACC, a precursor of ethylene, leads to a transient, more than 2-fold increase in HAI3, AHG1 and ABI2 expression 1 h after treatment. Cytokinin and auxin repress the transcription of the phosphatases compared to the untreated controls, indicating antagonistic regulation. Altogether, the expression of clade A PP2Cs is predominantly and clearly controlled by ABA.

Taken together, the transcriptome data reflect the functions attributed to clade A PP2Cs. There is a clear correlation between transcriptional alterations and ABA-mediated processes. Seed maturation, germination and response to drought, salt and osmotic stresses are mainly controlled by ABA. And all these response mechanisms are directly linked to PP2Cs from clade A as central components of the ABA-dependent signaling machinery.

2.2.2 RCAR transcriptional regulation

The direct regulation of PP2Cs by the ABA binding RCARs requires co-expression of both genes. The transcription of the phosphatases is extensively linked to ABA action (compare Fig. 21). In order to identify a similar control for the RCAR genes, their expression patterns were monitored. In this context the RCAR transcriptional response to ABA and other plant hormones is of particular interest.

Transcriptomic data available from AtGenExpress database (Schmid et al., 2005) show a reciprocal regulation of RCAR proteins compared to that of the PP2Cs (Fig. 22).

treatment time [h]	control	ACC [10 µM] 0,5	1	3	control	Zeatin [1 µM] 0,5	1	3	control	MeJA [10 µM] 0,5	1	3
RCAR1	0,022	0,92	0,91	1,14	0,022	1,00	0,76	1,01	0,022	0,54	0,63	0,78
RCAR2	0,032	1,06	1,10	0,98	0,032	1,08	1,24	0,92	0,032	1,27	1,39	1,55
RCAR3	0,093	1,08	1,19	1,21	0,093	0,98	0,74	0,85	0,093	1,21	1,00	1,39
RCAR8	0,135	0,92	1,45	1,25	0,135	0,54	0,78	0,77	0,135	0,62	0,84	0,91
RCAR9	0,036	1,10	1,02	1,22	0,036	0,83	1,10	0,99	0,036	0,60	0,57	0,72
RCAR10	0,367	0,93	0,94	0,98	0,367	0,89	0,94	0,93	0,367	0,98	0,89	0,85
RCAR11	0,133	0,98	0,94	0,86	0,133	1,07	1,07	1,05	0,133	0,87	0,78	0,70
RCAR12	0,044	1,08	0,90	0,84	0,044	1,06	0,97	1,19	0,044	0,99	0,86	1,19
RCAR13	0,005	1,43	1,47	1,45	0,005	1,01	0,88	1,89	0,005	1,51	1,04	1,31
RCAR14	0,010	1,46	1,20	0,87	0,010	0,90	0,80	1,92	0,010	1,30	1,02	1,08

treatment time [h]	control	IAA [1 µM] 0,5	1	3	control	ABA [10 µM] 0,5	1	3	control	GA3 [1 µM] 0,5	1	3
RCAR1	0,022	0,77	0,76	1,14	0,022	1,45	1,92	1,30	0,022	0,89	0,66	1,07
RCAR2	0,032	0,96	1,03	0,98	0,032	1,03	1,65	0,96	0,032	0,93	0,96	0,88
RCAR3	0,093	0,88	0,96	0,93	0,093	0,93	0,71	0,24	0,093	1,02	0,98	0,97
RCAR8	0,135	1,01	1,38	1,02	0,135	0,24	0,18	0,18	0,135	0,79	1,23	0,79
RCAR9	0,036	0,78	1,00	0,64	0,036	0,23	0,19	0,11	0,036	0,82	1,36	0,99
RCAR10	0,367	1,01	0,95	0,66	0,367	0,31	0,06	0,03	0,367	1,01	1,09	0,98
RCAR11	0,133	0,91	0,81	0,49	0,133	0,67	0,50	0,24	0,133	1,05	1,01	1,11
RCAR12	0,044	0,90	0,72	0,60	0,044	0,73	0,43	0,39	0,044	1,04	0,86	0,94
RCAR13	0,005	0,84	1,05	1,17	0,005	1,39	1,31	2,03	0,005	0,72	0,93	2,45
RCAR14	0,010	0,98	0,80	1,18	0,010	1,09	0,98	1,11	0,010	0,97	0,65	1,97

change in expression [x-fold]

0,10	0,33	0,66	1,00	3,33	6,66	10,00

<10x down-regulated up-regulated **>10x**

Fig. 22: Expression of RCAR genes after hormone treatment. The expression of ten RCAR genes relative to untreated controls is shown at different time points (0-3 h) after ACC, Zeatin, MeJA, IAA, ABA and GA3 treatment. Transcript abundance relative to ubiquitously expressed reference gene UBQ10 is indicated by the ratio given in the control column. Intensities of blue and orange indicate down- and up-regulation, respectively.

Plant hormone-mediated transcriptional regulation of the RCAR genes is visualized in Fig. 22. RCAR4, RCAR5, RCAR6 and RCAR7 are not included in this analysis because there is currently no data available for these genes. In the available datasets, the alterations in transcript levels for the RCARs are minor in response to most of the hormones tested. For instance, ACC, zeatin and GA3 cause slight down-regulation of some RCAR proteins. MeJA leads to a more than 1,5-fold decrease of RCAR1, RCAR8 and RCAR9. The other RCARs are not significantly affected. A similar down-regulation elicited by IAA can be observed for RCAR9, RCAR10, RCAR11 and RCAR12. Solely the treatment of the seedlings with exogenous ABA causes a specific and strong down-regulation of all RCARs except

RCAR1, RCAR2, RCAR13 and RCAR14. The decrease in transcript can be observed already after 0,5 h. For RCAR3, RCAR8, RCAR9, RCAR10 and RCAR11 the down-regulation is more than 4-fold after 3 h of ABA. A similar fashion of transcript regulation is observed after challenging the seedlings with hyperosmolarity, salt or drought (data not shown). Concordantly to the hormone-related data set, also during osmotic and salt stress RCAR2 and RCAR13 are transiently up-regulated in the roots (data not shown).

Nevertheless, the expression of most of the RCAR proteins is negatively controlled by ABA-dependent signaling. Comparison of the data retrieved from the gene expression databases illustrates that PP2Cs and RCARs are inversely correlated at the transcriptional level.

2.3 PP2Cs in the control of ABA downstream signaling

RCAR-dependent inhibition of PP2C function constitutes the major molecular switch in ABA-dependent signaling. Besides spatio-temporal differences in expression patterns, this mechanism is based on the different affinities of RCARs and PP2Cs for binding each other. Furthermore the different co-receptor complexes exhibit different affinities for the ligand, usually ABA (Szostkiewicz et al., 2010). As final output of this ABA receptor-PP2C-relay the signal transduction governing the comprehensive physiological responses is either retained or facilitated. Upon perception of the phytohormone, downstream targets are released from the control of the PP2Cs. Among the targets there are several protein kinases. These protein kinases belong to different subgroups, such as the CBL-regulated CIPKs, the Ca^{2+}-regulated CPKs or the ABA signaling-related SnRK2s.

2.3.1 PP2C-CIPK interaction

CIPK-family proteins are involved in Ca^{2+} signal integration (Batistic and Kudla, 2004). This protein kinase family in Arabidopsis comprises 26 proteins (Kolukisaoglu et al., 2004), some of which are reported to be involved in the control of transporter activity, ion homeostasis or salt stress signaling (Batistic and Kudla, 2009; Luan et al., 2009; Yu et al., 2007). Among these protein kinases there are known interaction partners of clade A PP2Cs (Guo et al., 2002; Ohta et al., 2003). Furthermore, an antagonistic functional relation between the clade A phosphatases and CIPKs in the regulation of a potassium channel has been proposed (Lee et al., 2007).

Based on the previously reported cases of binary interaction, the complement of all possible PP2C-CIPK combinations were to be analyzed in a targeted Y2H approach. Therefore, AD-fusions of all nine

clade A PP2Cs were tested against BD-fusions of CIPK-proteins for activation of the His-autotrophy reporter system. A compilation of exemplary combinations is shown in Fig. 23.

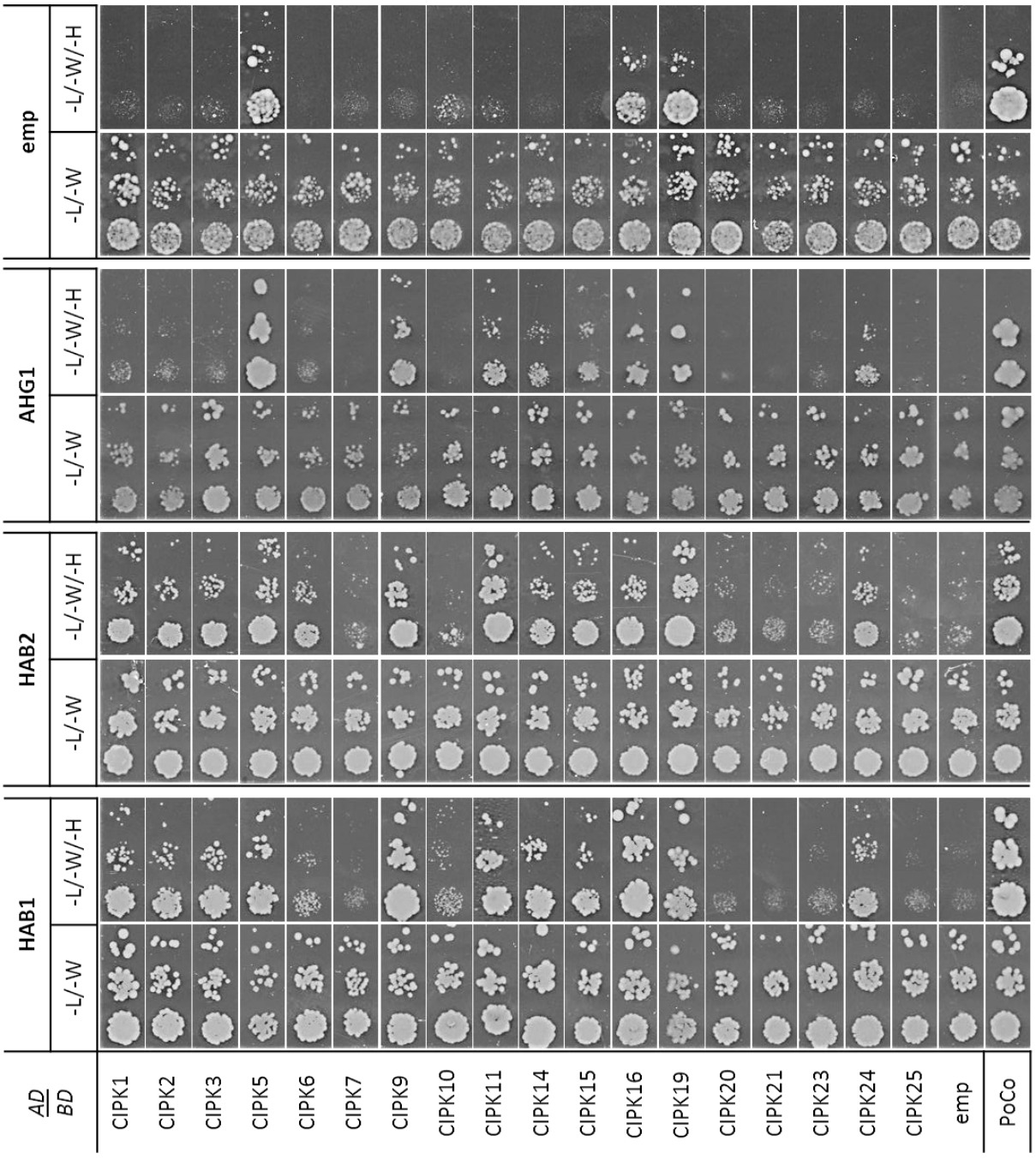


Fig. 23: Interaction of PP2Cs and CIPKs in the Y2H-system. Exemplary combinations of HAB1, HAB2, AHG1 phosphatases and empty AD vector (emp) with a random selection of CIPKs are spotted as dilution series. The yeast cells were incubated for 9 d and selected for His-autotrophy as a read-out for interaction. Growth on double selection (-L/-W) reflects positive dual-transformation and growth on triple selection (-L/-W/-H) reflects positive interaction. Empty vector (emp) combinations serve as negative controls. Positive control (PoCo) combinations are HAI3/RCAR3, ABI2/RCAR6, HAI3/RCAR7 and HAI3/RCAR8 from left to right.

The Y2H experiments show a characteristic pattern of PP2C-CIPK complex formation (Fig. 23). In order to identify constructs with reporter auto-activation the proliferation of clones harboring combinations of empty AD vectors (emp) and CIPK BD-fusions was monitored under selective conditions (-L/-W/-H plates). For instance CIPK5, CIPK16 and CIPK19 exhibited His-autotrophic growth in combination with the empty pGAD vector indicating auto-activation. On the other hand true binary interactions could be observed for CIPK1, CIPK2, CIPK3, CIPK9, CIPK11, CIPK14, CIPK15 and CIPK24 with HAB1 PP2C. Highly homologous HAB2 showed a clear overlap in interaction preferences. Additional to the CIPKs identified as HAB1 interactors, HAB2 also interacts with CIPK6. Phosphatase AHG1 interacted with CIPK9, CIPK11, CIPK14, CIPK15 and CIPK24 in the assays presented. These analyses exemplify the distinct pattern of interaction between clade A PP2Cs and CIPKs.

After positive testing for auto-activation CIPK5, CIPK12, CIPK13, CIPK16, CIPK18 and CIPK19 were excluded from the analyses (compare Fig. 23). The remaining CIPKs were screened for interaction with the clade A PP2Cs using the His-autotrophy reporters system. To corroborate these experiments the adenine (Ade)-autotrophy reporter of the AH109 yeast cells was used. Exemplary combinations of PP2Cs and CIPKs were investigated for interaction-dependent proliferation on selection medium deficient for adenine (-L/-W/-A).

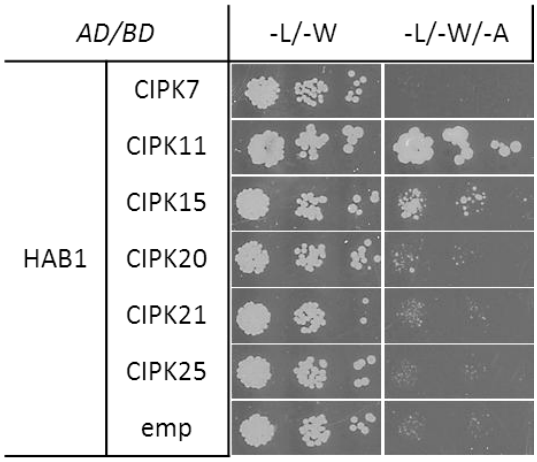


Fig. 24: Interaction of HAB1 and CIPKs. Exemplary combinations of HAB1 with a selection of CIPKs are spotted as dilution series. The yeast cells were selected for Ade-autotrophy as a read-out for interaction. Growth on double selection (-L/-W) reflects positive dual-transformation and growth on triple selection (-L/-W/-A) reflects positive interaction. An empty vector (emp) combination serves as negative control.

Also a second Y2H reporter system substantiates specific coupling of PP2Cs and CIPKs. These experiments provide similar results as the His-autotrophy assays. HAB1 exhibits strong interaction with CIPK11 and weak interaction with CIPK15 (Fig. 24). CIPK7, CIPK20, CIPK21 and CIPK25 do not interact with HAB1 in yeast. Taken together, these analyses in the yeast system circumstantiate a

versatile pattern of interaction between different PP2Cs and CIPKs. An overview of the complement of possible combinations analyzed in yeast is presented in Fig. 25.

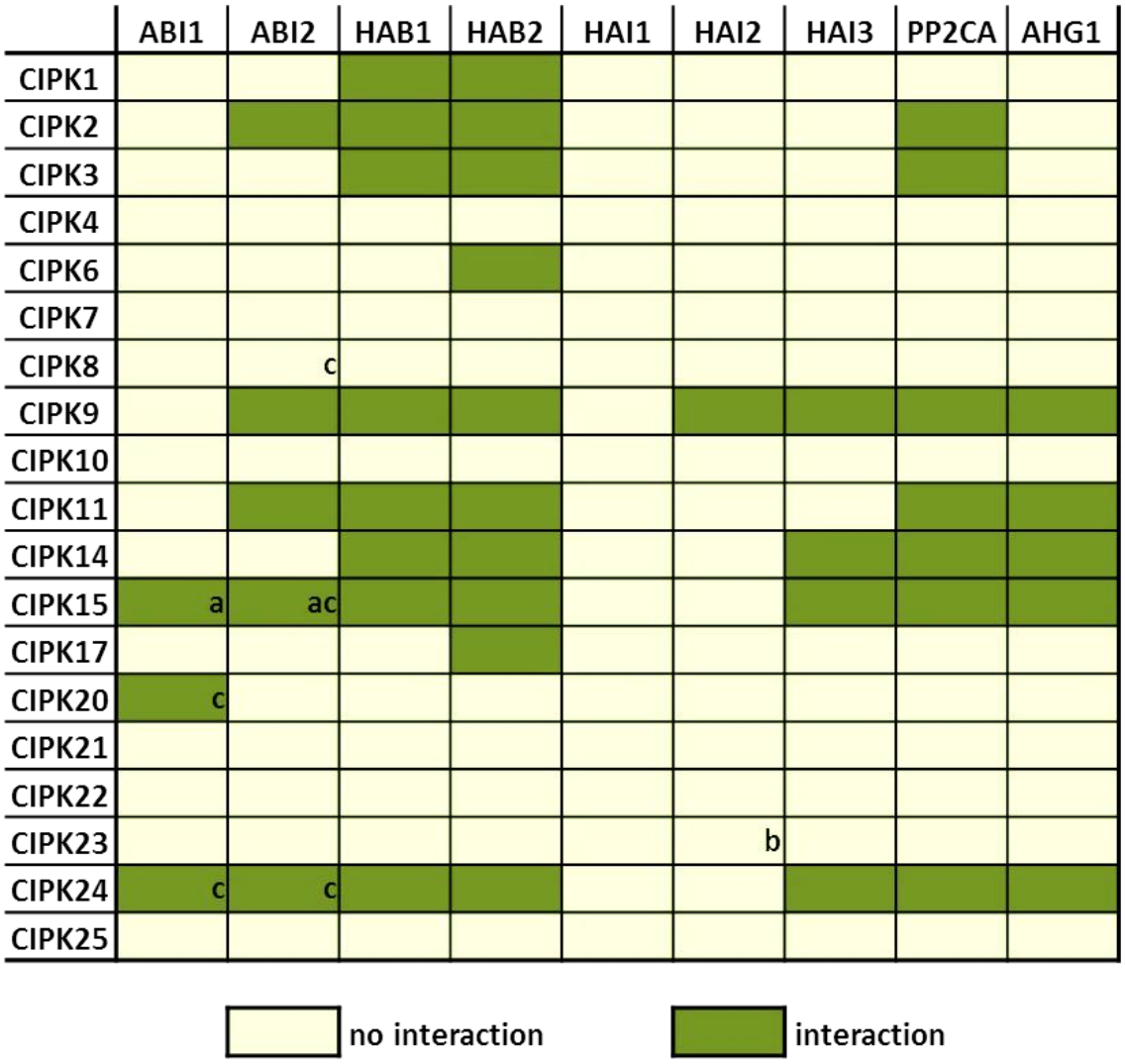


Fig. 25: Summary of interactions of PP2Cs and CIPKs. The complement of combinations tested for interaction is presented as a matrix. PP2C and CIPKs are fused to Gal4 AD and BD, respectively. Dark shading indicates interaction and lowercase letters mark previously reported interactions according to Guo et al. (2002)[a], Lee et al. (2007)[b] and Ohta et al. (2003)[c].

Protein-protein interaction was determined for a compilation of 225 possible interactions of the nine clade A PP2Cs and 25 CIPKs from Arabidopsis (Fig. 25). Auto-activating CIPK fusions are not included in the summary. The constructs used in these studies harbor the wt CDS of the CIPKs in pGBT Y2H vectors and were kindly provided by J. Kudla, University of Münster, Germany. The conducted assays largely confirmed the interactions reported earlier: CIPK15, CIPK20 and CIPK24 showed interaction with ABI1, consistent with the results from Guo et al. (2002) and Ohta et al. (2003). The interaction of

ABI2 with CIPK15 and CIPK24 also confirmed previous reports (Guo et al., 2002; Ohta et al., 2003). An association of ABI2 with CIPK8 reported by Ohta et al. (2003) was not observed. Lee et al. (2007) showed HAI2-CIPK23 interaction, which is not confirmed by the present Y2H data.

Nevertheless, the combinatorial interaction analyses revealed several interactions not documented so far. CIPK1, CIPK2, CIPK3, CIPK9, CIPK11, CIPK14, CIPK15 and CIPK24 interacted with two or more of the clade A PP2Cs in this setup. For CIPK6 and CIPK17 only single interactions with HAB2 were detected. HAI2 PP2C solely interacted with CIPK9. HAI1 did not show interaction with any of the CIPKs in yeast under the present conditions.

Interference of PP2Cs and CIPKs *in vivo*

For further analyses, the PP2C-interacting CIPKs were sub-cloned into a suitable plant expression vector (see 4.2.12 for details). The direct interaction of PP2Cs and the protein kinases suggests an involvement in ABA-dependent processes. To study CIPK-related effects on ABA signal transduction, a transient protoplast expression system was utilized. Subsequent stable, ectopic expression *in planta* could be informative about the function *in vivo*.

Besides their interaction with PP2Cs ABI1 or ABI2, CIPK15 and CIPK20 are involved in ABA-dependent processes. Knockout mutants of CIPK15 show ABA hypersensitive phenotypes in germination, stomatal response and gene regulation, while over-expression lines are insensitive to ABA in stomata regulation (Guo et al., 2002). Contrastingly, CIPK20-silenced plants are insensitive to ABA, while ectopic protein levels conferred hypersensitivity (Gong et al., 2002). First experiments with over-expression of the recombinant wt CIPK15 and CIPK5 did not show a significant effect on ABA signal transduction in protoplasts (data not shown). This might be explained by the mechanism of CIPK activation (compare 1.4.3). To circumvent the necessity of Ca^{2+}/CBL-dependent activation of CIPKs, constitutively active (CA) versions were generated by site directed mutagenesis (SDM) according to Liu and Naismith (2008). Specifically modified oligonucleotides were used to prime PCR-based plasmid amplification yielding mutated copies of the vector (see 4.2.6 for details). Using this technique a conserved threonine (T) in the activation loop of the protein kinase was changed to an aspartate (D). This point mutation simulates phosphorylation and renders the CIPK active without the obligate binding of the CBL sensory protein (Gong et al., 2002; Guo et al., 2001; Pandey et al., 2008; Tripathi et al., 2009; Xu et al., 2006).

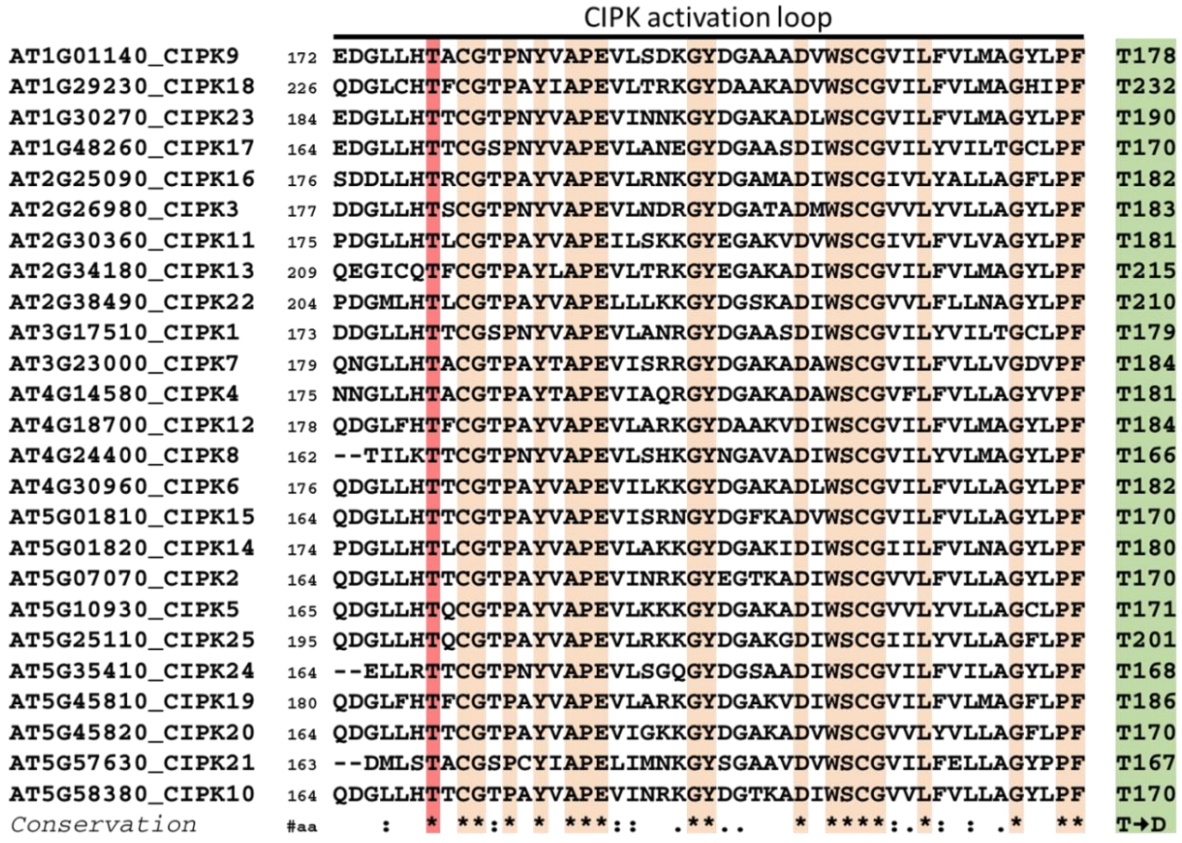


Fig. 26: Protein sequence alignment of 25 Arabidopsis CIPKs. The conserved T residue (highlighted in red) in the activation loop region is mutated to D to generate a constitutively active (CA) version of the kinase. Asterisks mark conserved residues. The position of the mutated threonine is highlighted in green.

The sequences of CIPK proteins are aligned in Fig. 26. An amino acid exchange in a conserved residue of the kinase activation loop was introduced for the generation of CA CIPKs. The important threonine (T) is conserved in all Arabidopsis CIPKs. Its replacement with D mimics phosphorylation and leads to kinase activation. The SDM reaction was successfully performed for the 25 CIPKs tested. To visualize the effect of active CIPKs on ABA signaling, the pRD29B::LUC ABA-responsive reporter system in protoplasts was used (see 4.2.11). In wt Col mesophyll protoplasts a selection of CIPKs were ectopically expressed. The CIPKs tested in protoplasts include CIPK2, CIPK9, CIPK11, CIPK14, CIPK15, CIPK20 and CIPK24 interacting with PP2Cs in Y2H assays. CIPK15, CIPK20 and CIPK24 have been reported to be directly connected to ABA-dependent processes (Cheng et al., 2004; Gong et al., 2002; Guo et al., 2002). CIPK9, CIPK11, CIPK14 are involved in potassium homeostasis (Pandey et al., 2007), the regulation of pH gradients (Fuglsang et al., 2007) and sugar signaling (Lee et al., 2005), respectively. The interaction of these CIPKs with the PP2Cs might interconnect these processes to ABA signaling. Also non-interacting CIPK8 and CIPK21 were tested. Furthermore, CIPK5, CIPK12, CIPK13, CIPK18 and CIPK19, which showed Y2H auto-activation, were included in the protoplast experiments. Constructs with CIPK wt sequences were used as controls.

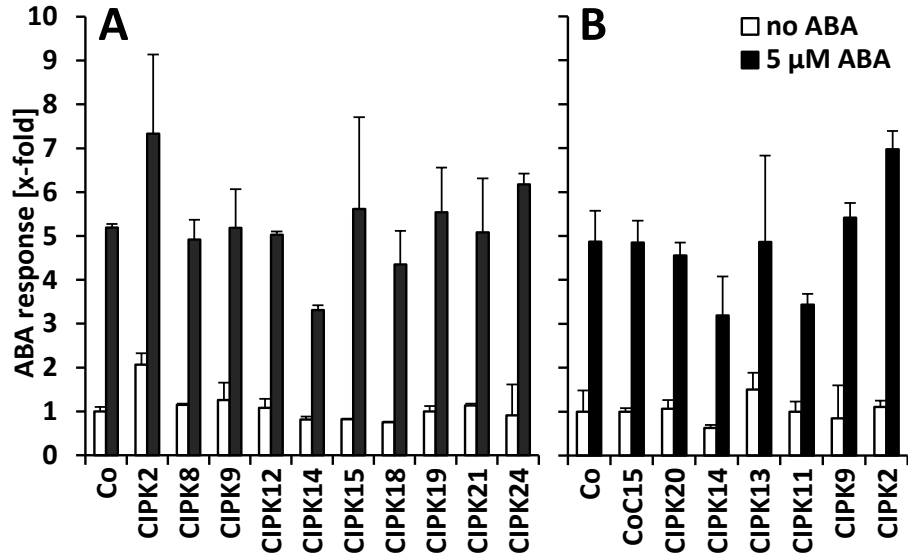


Fig. 27: CIPKs in Col wt protoplasts. The effect of ectopic CIPK expression on ABA response in two exemplary experiments is shown as x-fold signal induction relative to untreated control (Co). (A) CA versions of ten different CIPKs were tested for reporter induction. (B) CA versions of six different CIPKs were tested for induction of the ABA-responsive reporter. The inactive wt version of CIPK15 (CoC15) was used as additional control. Samples were incubated for 16 h without or with 5 µM ABA. For each effector 1 µg of CIPK plasmid DNA was transfected. Mean GUS activities are 0,135 (± 0,012) and 0,180 (± 0,058) for experiments A and B, respectively.

The general effect of ectopic CIPK expression on ABA-dependent gene regulation is shown in two exemplary protoplast assays (Fig. 27). ABA response reflects the induction of the ABA-specific pRD29B::LUC reporter activation relative to a constitutive GUS standard. In these experiments with Col wt protoplasts ABA causes approximately a 5-fold induction of the reporter. Co-expression of most of the constitutively active CIPKs did not cause a significant change in the ABA response. An unmodified and hence inactive wt version of CIPK15 (CoC15) shows reporter activation equal to empty vector samples (Co) and serves as additional control (Fig. 27B). CIPK2 elicits a minor increase in several experiments. On the other hand CIPK11 and CIPK14 slightly but reproducibly repress the reporter activity. In order to minimize the effect of endogenous ABA signaling, a series of similar experiments was conducted in protoplasts harvested from the ABA deficient mutant *aba2* (Schwartz et al., 1997). This biosynthesis mutant only contains minimal levels of ABA and responds sensitively to external ABA.

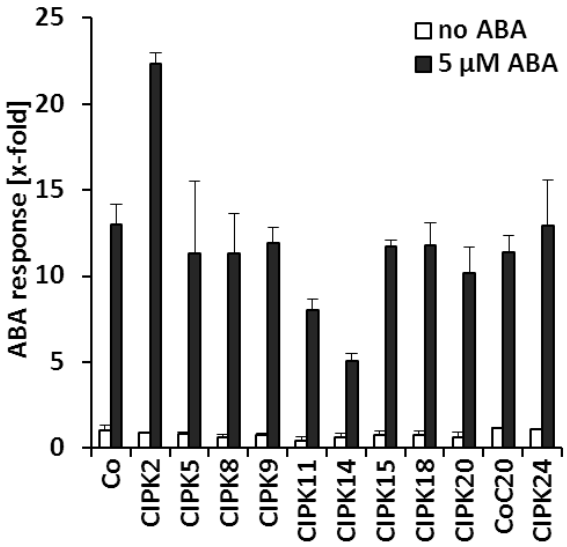


Fig. 28: CIPKs in *aba2* protoplasts. The effect of ectopic CIPK expression on ABA response is shown as x-fold signal induction relative to untreated control (Co). CA versions of ten CIPKs were co-transfected and compared to the induction of the empty vector (Co) and inactive wt CIPK20 (CoC20) controls. Samples were incubated for 16 h without or with 5 µM ABA. For each effector 1 µg of CIPK plasmid DNA was transfected. Mean GUS activities for these samples are 1,836 (± 0,241) RFU/sec.

In an ABA deficient mutant background the effect of CIPK overexpression is similar to that in Col wt (Fig. 28). With the exception of CIPK2, CIPK11 and CIPK14 the ABA response remains unchanged in the presence of active CIPKs. Co-expression of CIPKs results in values equal to inactive wt CIPK20 (CoC20) and empty vector (Co) controls. Nevertheless, the induction seen for CIPK2 is more pronounced than in wt Col protoplasts. After induction with 5 µM ABA CIPK2 further raises ABA response by a factor of 1,7 relative to the control. CIPK11 and CIPK14 significantly constrict ABA-dependent signal transduction. Ectopic levels of CIPK11 and CIPK14 reduce ABA-induced response by a factor of 1,6 and 2,6, respectively. This repression can be observed also in the absence of the ABA stimulus. Both protein kinases reduce ABA-dependent signaling to around 50 % of the untreated empty vector control (Co).

Thus, the experiments with the CA versions of these protein kinases exhibit a small but reproducible effect on the regulation of ABA-dependent gene expression. Further protoplast experiments were performed to analyze this effect in detail.

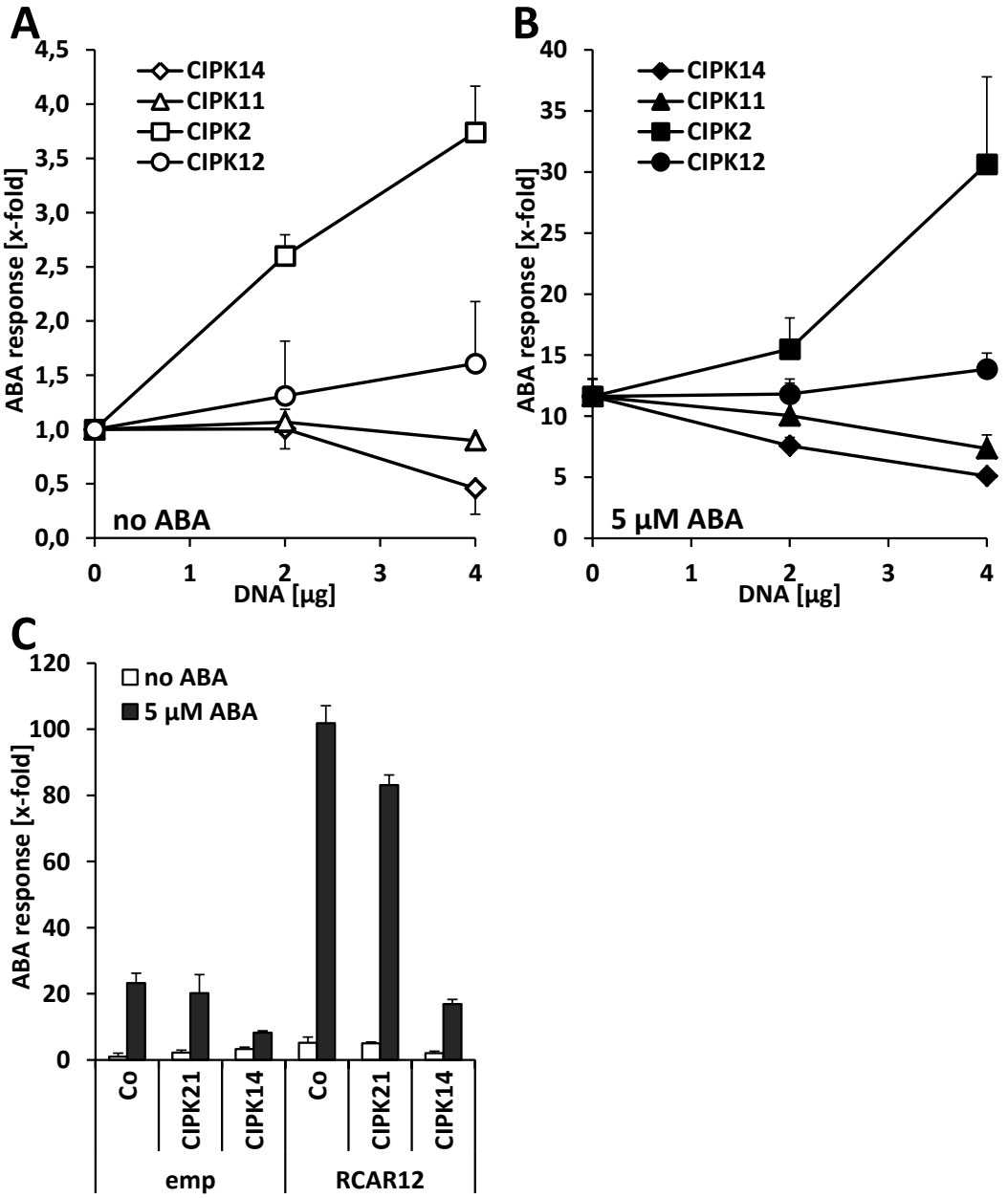


Fig. 29: CIPK function in *aba2* protoplasts. The effect of ectopic CIPK expression on ABA response is shown as x-fold signal induction relative to untreated control (Co). Different amounts of CIPK2, CIPK11, CIPK12 and CIPK14 effector plasmid were co-transfected to study ABA response alterations in the absence (A) or presence (B) of exogenous ABA. (C) CIPK21 and CIPK14 effector plasmids (6 µg each) were co-transfected with the empty vector (emp) or RCAR12 (1 µg each) to study their effect on RCAR-dependent ABA response. Samples were incubated for 16 h (A,B) or for 20 h (C) without (open symbols/bars) or with 5 µM ABA (black symbols/bars). Mean GUS activities are 0,554 (± 0,131) RFU/sec (for A/B) and 0,806 (± 0166) RFU/sec (for C).

The inductive effect of CIPK2 and the repressive effects of CIPK11 and CIPK14 on ABA-dependent gene regulation are presented in Fig. 29. With increasing concentrations of CIPK14 and CIPK11 ABA-dependent signaling is reduced, particularly in the presence of ABA (Fig. 29B). With 4 µg of CIPK DNA transfected, both protein kinases inhibited signal transduction for more than 30 % compared to ABA-treated control. Contrary to this, CIPK2 induces ABA reporter activity in a dose-dependent manner.

Particularly, in a non-induced state ABA response is induced by a factor of 2,5 (Fig. 29A). A similar level of induction was observed at higher concentrations of the CIPK CA version in the presence of ABA (Fig. 29B). CIPK12 serves as a control in this context because it does not significantly influence reporter activation, nor in the presence or absence of the ligand. The effect of CIPK14 and CIPK21 on RCAR-dependent ABA signal transduction is illustrated in Fig. 29C. Co-expression CIPK21 does not significantly alter reporter activity and can be considered as a control. CIPK14 blocks ABA signaling. This inhibitory effect on ABA reporter activity occurred also in the presence of RCAR12, whose over-expression triggers strong ABA-dependent reporter activation.

The results gathered in the heterologous yeast system and the effects seen in protoplasts indicate a connection of a subset of CIPK proteins to ABA-controlled signaling. The distinct effects of constitutive activation of CIPK11, CIPK14, and CIPK2 observed might be due to interference with PP2C function. Protoplast experiments were performed to assess the combined effect of CIPKs and PP2Cs on ABA-dependent signaling.

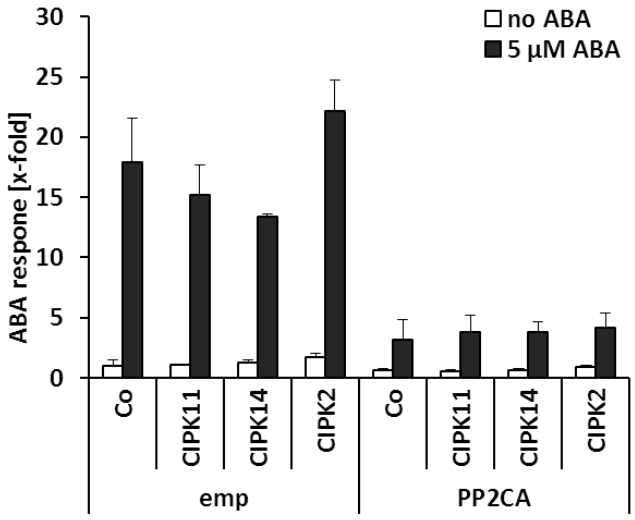


Fig. 30: Combined CIPK-PP2C action in ABA signaling. The effect of CIPK-PP2C co-expression on ABA response in protoplasts is shown as x-fold signal induction relative to untreated empty vector (emp) control (Co). 2 µg of CIPK11, CIPK14 and CIPK2 CA effector plasmid were co-transfected with 0,2 µg of empty vector (emp) or PP2CA. Samples were incubated for 18 h without or with 5 µM ABA. Mean GUS activity of these samples is 0,460 (± 0,132) RFU/sec.

The Y2H interaction data suggest a functional relation of some PP2Cs and a subset of CIPKs. For example PP2CA is clearly interacting, amongst others, with CIPK2, CIPK11 and CIPK14 in yeast (compare Fig. 25). To study the functional interplay, the PP2C and these CIPKs were co-expressed in protoplasts. Concomitant expression of CIPK2, CIPK11 and CIPK14 with PP2CA only marginally affected ABA response (Fig. 30). Expression of CIPK11 or CIPK14 alone causes a slight reduction, and

of CIPK2 alone a slight increase of ABA-dependent reporter activity (compare Fig. 28). In the presence of PP2CA the inhibitory effect of CIPK11 and CIPK14 is abolished, irrespective of an ABA induction. Reporter activity without exogenous ABA was induced by over-expression of CA CIPK2 also in the presence of PP2CA. The signal was enhanced by approximately 30 % compared to the samples with PP2CA alone. In the presence of exogenous ABA, there is no significant effect of CIPK2 on the ABA response.

Based on their interaction with PP2Cs in yeast and their effect on ABA-dependent gene regulation observed in protoplasts CIPK2, CIPK11, CIPK14 and CIPK20 were selected for further analyses. CA versions of CIPK2, CIPK11, CIPK14 and CIPK20 were introduced into Arabidopsis in order to elucidate the function of CA CIPKs *in planta* (Guo et al., 2004). Therefore the AscI expression cassette of the protoplast vectors was transferred to a binary vector system (4.2.10). Ectopic expression is driven by the CMV-derived 35S promoter. Eventually transgenic Col wt plants were generated via Agrobacterium-mediated transformation. For each CIPK of interest two versions were transferred into the plants. One vector contained the inactive wt version and the other one construct contained the point mutated CA CIPK. Transgenic lines carrying the empty vector construct alone were used as control. For each construct at least four independent transgenic lines were established. The seed material was amplified and the progeny was checked for the presence of the transgene. The different lines produced did not show any obvious growth defects or severe developmental phenotypes. However, for further characterization of these lines the expression levels of the transgenes need to be determined. Detailed analyses of ABA-related phenotypes may elucidate the relation of CIPK function and ABA signaling as well as the importance of PP2C-CIPK pairing *in planta*.

2.3.2 PP2C-CPK interaction

The range of signaling elements interfering with PP2Cs also includes CPKs. Some of these Ca^{2+}-sensing protein kinases are known to be interwoven with the ABA signal transduction network (Choi et al., 2005; Zhu et al., 2007; Zou et al., 2010). There are reports of mutual regulation of CPKs and the PP2Cs involved in ABA signaling (Geiger et al., 2011; Geiger et al., 2009). Among the kinases relevant for ABA signaling is CPK23. It was reported to be a positive regulator of ABA-signaling (Ma and Wu, 2007). Making use of the Y2H system, a physical interplay of clade A PP2Cs and CPK23 together with its closely related homolog CPK21 was tested. In the pBridge vector BD-fusions of different CPKs and variants thereof were generated. They were tested for interaction with AD-PP2C fusions in yeast.

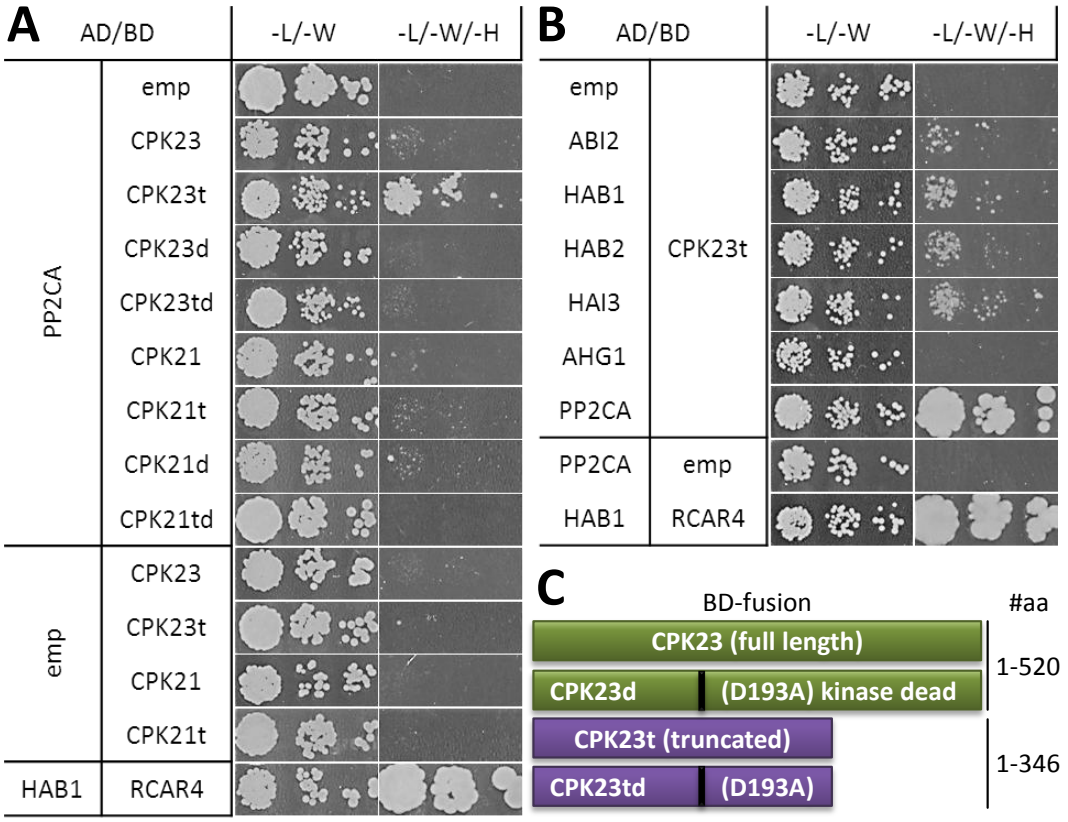


Fig. 31: PP2C-CPK interaction. The Y2H interaction of CPK21 and CPK23 with clade A PP2Cs was analyzed as dilution series spotting. The combinations were selected for His-autotrophy as read-out for interaction. (A) PP2CA was tested with different versions of CPK23 and CPK21. (B) The truncated version of CPK23 (CPK23t) was tested against several clade A PP2Cs. HAB1/RCAR4 and empty vector (emp) combinations serve as positive and negative controls, respectively (C) The different versions of CPK23 BD-fusions CPK23 full length, kinase dead full length (CPK23d), truncated constitutively active (CPK23t) and truncated kinase dead (CPK23td) are schematically depicted. The size of the respective proteins is indicated as number of amino acids (#aa).

Analyses of CPK23 and closely related, highly homologous CPK21 in the Y2H system revealed that full length version do not interact with clade A PP2Cs. Only the truncated version of $CPK23_{1-346}$ (CPK23t) interacts with PP2CA (Fig. 31A). The truncated version is deregulated due to the removal of the c-terminal autoinhibitory and regulatory domains (Harper et al., 1994; Huang et al., 1996; Ludwig et al., 2005). This constitutively active protein kinase interacts with PP2CA. Furthermore the interaction depends on the kinase activity, since the kinase dead version mutated at D193A (CPK23td) does not interact. Amongst the PP2Cs, the strongest interaction is observed for PP2CA. Other clade PP2Cs, namely HAI3, HAB1, HAB2 and ABI2 exhibit weaker, yet significant interaction (Fig. 31B). These assays substantiate an interaction of active CPK23 with clade A PP2Cs, notably PP2CA. For more detailed analyses the yeast transformants were also tested in the YLGA. The kinetics of interaction-dependent growth yield information about the strength of interaction.

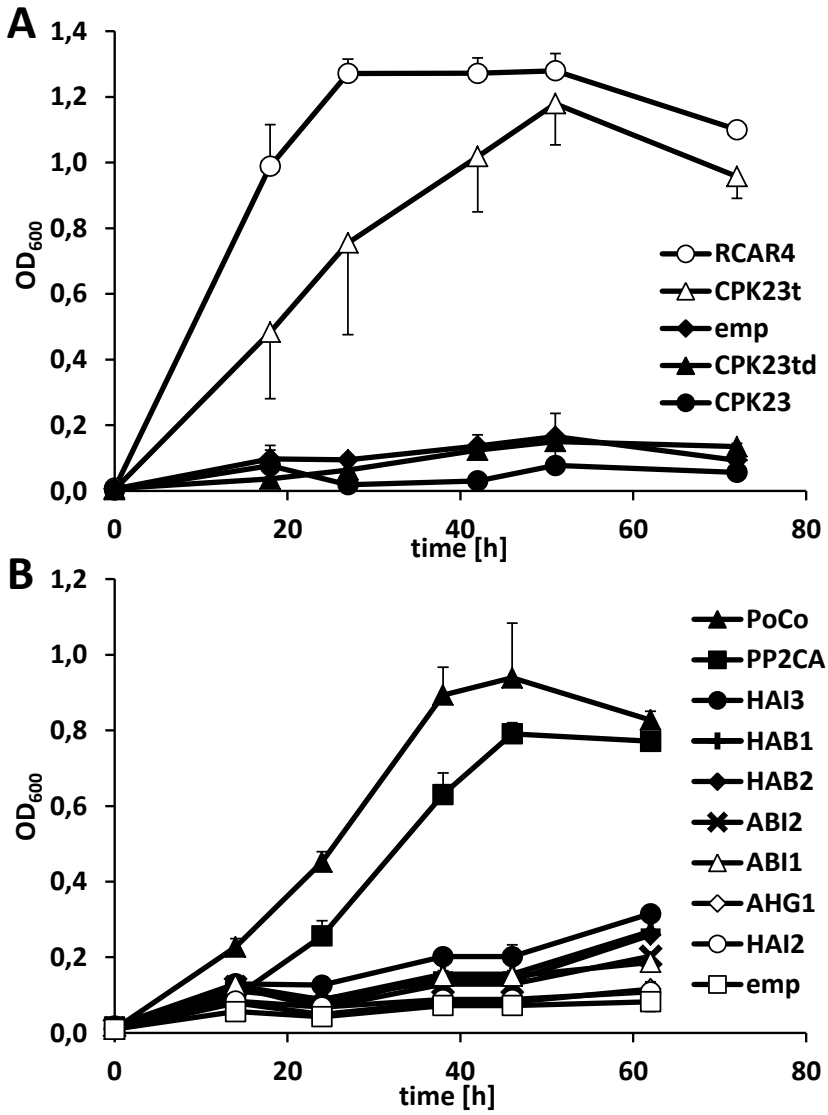


Fig. 32: PP2C-CPK interaction in liquid culture growth assay. Y2H interaction of CPK23 variants with clade A PP2Cs was analyzed. Yeast growth in triple selective medium is monitored over time. Values of OD_{600} reflect interaction-dependent His-autotrophy. Empty vector (emp) combinations serve as negative control. (A) Combinations of PP2CA with full length CPK23, truncated CPK23 (CPK23t) and a kinase dead truncated version (CPK23td) were assayed in liquid culture. PP2CA/RCAR4 combination serves as positive control. (B) Combinations of CPK23t with clade A PP2Cs were assayed in liquid culture. PP2CA/RCAR2 combination serves as positive control (PoCo). Values are means (± SD) of three independent transformant lines. OD_{600} of the samples at inoculation (t=0) are < 0,010.

The interaction seen for PP2CA and the truncated CPK23 version were confirmed by the Y2H liquid culture assays (Fig. 32). Only the constitutively active form of the kinase (CPK23t) interacted with PP2CA. The full length or the kinase dead version (CPK23 or CPK23td, respectively) did not proliferate in selective media (Fig. 32A). The interaction kinetics indicated interaction that is weaker compared to the positive control, which was a constitutively interacting combination of PP2CA and RCAR4. Furthermore, PP2CA exhibited the strongest interaction with CPK23t In comparison to the other clade A PP2Cs (Fig. 32B). HAI3 and HAB1 interaction with CPK23t was very weak and not sufficient to

completely activate His-autotrophy read-out under these experimental conditions. Interestingly, interaction of ABI1, AHG1 and HAI2 with the active version of the protein kinase was not sufficient to induce reporter activity. Taken together, the Y2H analyses provided clear evidence for a physical linkage of CPK23 to PP2CA.

Interference of CPK and RCAR signaling

Consequently, Y3H analyses were conducted to test for competitive interaction of CPKs and RCARs with PP2Cs. These experiments were designed to illustrate functional interference of CPKs with the central regulatory node of ABA signaling. AD-fusions of PP2Cs were tested against CPK-BD constructs carrying an inducible version of the third component, i.e. the RCAR protein. RCAR1, RCAR9 and RCAR11 genes were sub-cloned in the pBridge vector (from Clontech), namely into the MCSII, which is under the control of a methionine (M)-repressed promoter. Hence, co-expression of these proteins is controlled by the methionine levels in the media (Tirode et al., 1997). In the following Y3H analyses the RCAR proteins are induced to challenge CPK interaction. The three selected RCARs are members of the different subfamilies. The BD-fusion contains CPK23t for interaction studies.

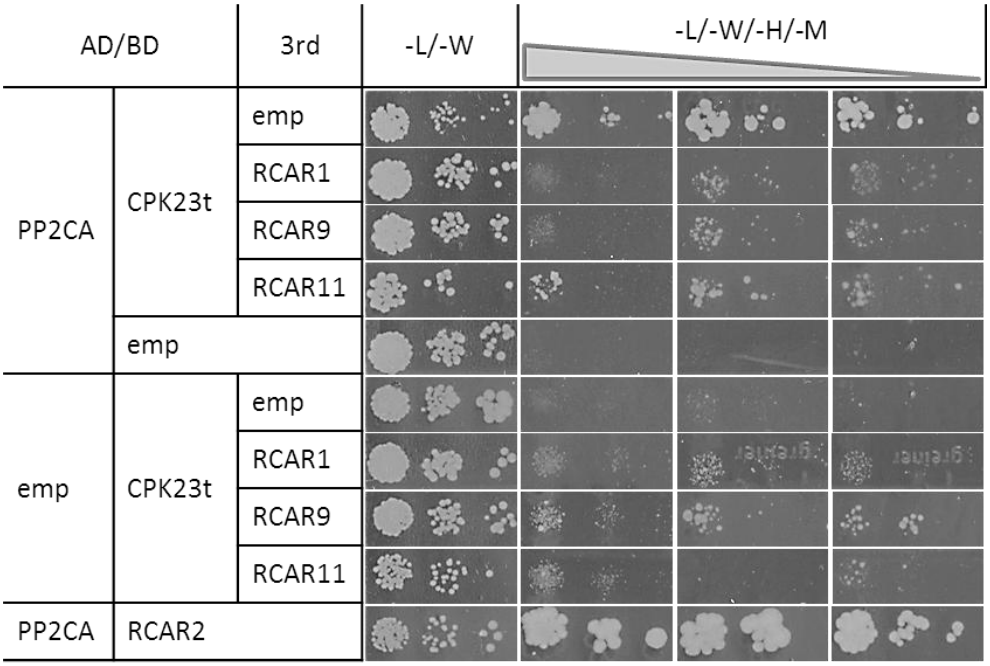


Fig. 33: Ternary complex of PP2C, CPK and RCAR. In a Y3H approach PP2CA competitive interactions with CPK23 or RCARs were spotted as dilution series. Growth on selective media (-L/-W/-H/-M) reflects interaction. Grey slopes indicate co-expression of the third protein component due to M-dependent promoter repression. M is supplemented to the selective media at 0, 0,15 and 1 mM, from left to right. Interaction of PP2CA with truncated CPK23 (CPK23t) was challenged by co-expression of RCAR1, RCAR9 and RCAR11 at different levels. PP2CA/RCAR2 and empty vector (emp) combinations serve as positive and negative controls, respectively.

Based on the interaction of PP2CA with the constitutively active CPK23, a possible interference of RCAR proteins was tested. The Y3H spottings in Fig. 33 show clear interaction of the phosphatase with CPK23t, when there was no third component expressed (emp). The co-expression of RCAR1 and RCAR9 at high levels completely abolished this interaction. In the case of RCAR11 even high levels of the third protein did not completely block PP2C-CPK interaction. With increasing methionine concentrations the levels of RCARs present in the yeast cells were repressed. But also at high methionine levels the interaction of PP2CA with CPK23t was not re-established indicating an efficient competition of all three RCARs investigated. The empty vector controls of CPK23t alone do not show any reporter activation. The co-expression of RCAR1 and RCAR9 as a third component causes some background reporter activity in the empty AD combinations.

To complete the Y3H analyses on CPK-RCAR competition, BD-fusions of RCARs were tested for PP2C interaction in the presence or absence of CPK23. Therefore, CPK23 was conditionally expressed as active version (CPK23t) or kinase dead version (CPK23td) as a third component.

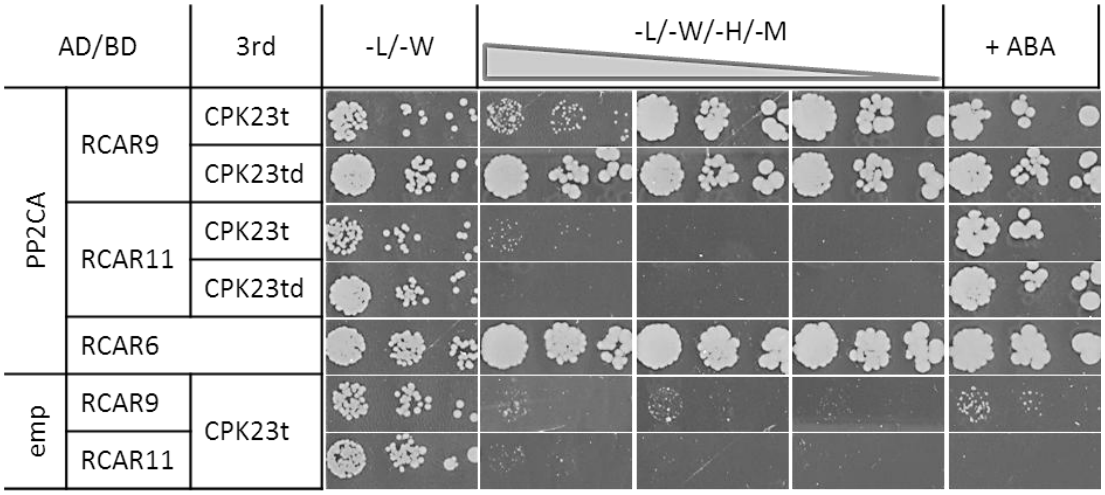


Fig. 34: Ternary complex of PP2C, RCAR and CPK. PP2CA interactions with RCAR9 and RCAR11 were challenged by co-expression of truncated (CPK23t) and kinase dead truncated CPK23 (CPK23td). Growth on selective media (-L/-W/-H/-M) reflects interaction. Grey slopes indicate co-expression of the third protein component due to M-dependent promoter repression. M is supplemented to the selective media at 0, 0,15 and 1 mM, from left to right. ABA-dependency of PP2C-RCAR interaction is visualized in triple selective media (-L/-W/-H) supplemented with 30 µM ABA (+ABA). PP2CA/RCAR6 and empty vector (emp) combinations serve as positive and negative controls, respectively.

CPK23 influences the interaction of PP2CA with distinct RCAR proteins (Fig. 34). Low methionine concentrations inducing high CPK23t expression significantly reduce the interaction of RCAR9 with PP2CA. Smaller amounts of CPK23 were not sufficient to disturb PP2CA-RCAR9 interaction in these assays. Strikingly, the decrease of PP2CA-RCAR9 docking caused by CPK23 was dependent on a

functional kinase. The kinase dead version (CPK23td) was not able to interrupt the coupling of the phosphatase and RCAR9. Fig. 34 also confirms the ABA-dependent interaction of PP2CA and RCAR11. In the absence of the ligand the PP2C does not interact with RCAR11. A co-expression of CPK23 did not show any effects. The formation of the PP2C-RCAR11 complex was stabilized by applying exogenous ABA (+ABA). The present experiments for CPK23 provide evidence that only a truncated, constitutively active and functional version interacts with PP2CA. Furthermore, the active form of the protein kinase interferes with the binding of the directly associated PP2C signaling regulators RCARs.

Interference of CPK and SnRK2 signaling

SnRK2 proteins are major downstream targets of PP2C-dependent inhibition (Hirayama and Umezawa, 2010). They positively regulate ABA signal transduction (Fujii and Zhu, 2009; Kobayashi et al., 2005). Direct physical interaction with the PP2Cs has been shown for SnRK2s (Nishimura et al., 2010; Umezawa et al., 2009) and the aforementioned CPKs (Geiger et al., 2011). Functional interplay of these two types of protein kinases might converge at the PP2C relay. In Y3H competitive interaction studies the role of CPK23 in PP2C-dependent SnRK2 inhibition was assessed. Furthermore, the effects of SnRK2 proteins on the CPK23-PP2C interaction could be analyzed. Therefore, the CPK23 and the SnRK2s were alternately cloned into pBridge vectors as BD-fusions or as conditionally expressed third component. As controls a kinase dead version of SnRK2.6, i.e. mutated at D160A (SnRK2.6d) and the kinase dead version CPK23td were included in the analyses.

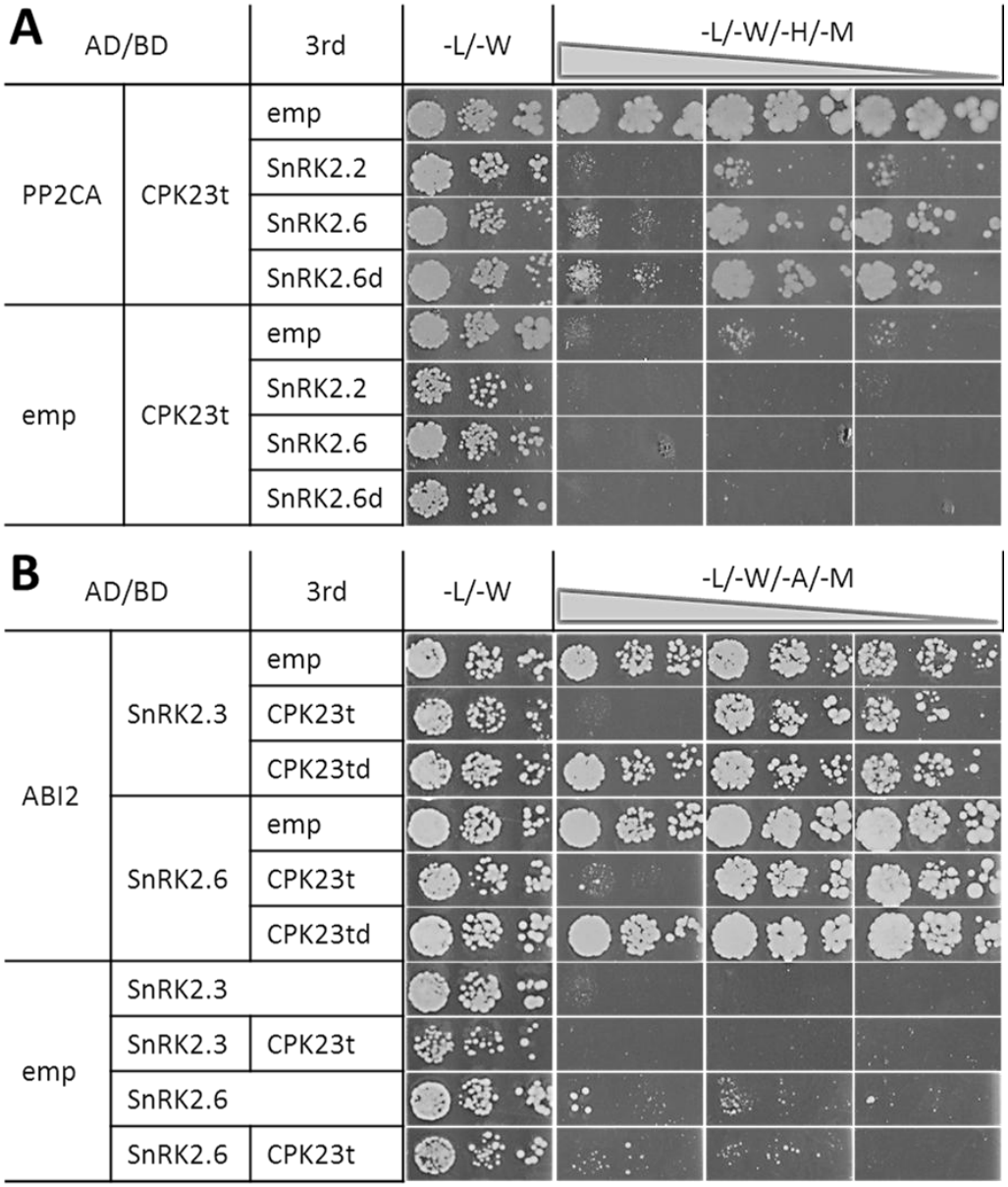


Fig. 35: Ternary complex of PP2C, CPK and SnRK2. In a Y3H approach PP2CA and ABI2 were analyzed for competitive interactions with CPK23 or SnRK2s as dilution series spottings. Growth on selective media (-L/-W/-H/-M or -L/-W/-A/-M) reflects interaction. Grey slopes indicate co-expression of a third protein due to M-dependent promoter repression. M is supplemented to the media at 0, 0,15 and 1 mM, from left to right. Empty vector (emp) combinations serve as negative controls. (A) PP2CA interaction with truncated CPK23 (CPK23t) was challenged by co-expression of SnRK2.2, SnRK2.6 and kinase dead version of SnRK2.6 (SnRK2.6d). (B) ABI2 interactions with SnRK2.3 and SnRK2.6 were challenged by co-expression of truncated and kinase dead truncated versions of CPK23 (CPK23t and CPK23td). Plates were incubated for 10 d (A) and 9 d (B).

The analyses in the heterologous yeast system confirm PP2CA interaction with CPK23t (Fig. 35). This coupling was inhibited by the co-expression of SnRK2.2 and also by SnRK2.6 at high levels (Fig. 35A). Lower levels of SnRK2.2 and particularly SnRK2.6 (and SnRK2.6d) were not sufficient to interrupt PP2CA-CPK23 interaction. The point mutated kinase dead version of SnRK2.6 (SnRK2.6d) has the same effect as the wt SnRK2.6 in these Y3H assays. Hence, the competitive effect of SnRK2.6 seems

to be independent of its kinase activity. In contrast to this the experiments in Fig. 35B showed that only active CPK23 (CPK23t) but not the mutated version (CPK23td) was able curtail the interaction of PP2C ABI2 and SnRK2.3 and SnRK2.6. The strong interaction seen for ABI2 with SnRK2.3 and SnRK2.6 confirmed previous reports by Umezawa et al. (2009). High levels of active CPK23t but not CPK23td diminished the formation of the PP2C-SnRK2 complex. This CPK23-mediated inhibition of PP2C-SnRK2 coupling clearly depended on its kinase function. Lower concentrations of CPK23 did not interrupt PP2C-SnRK2 interaction. Taken together, these results show that SnRK2s can displace CPK23 and interrupt its interaction with PP2CA. On the other hand, PP2C binding to its downstream factors SnRK2s can be inhibited by active CPK23.

2.3.3 PP2C-ABF interaction

Besides their interaction with CIPKs, SnRK2s and CPKs cellular targets such as ion channels, PP2Cs were shown to directly interact with transcription factors (Himmelbach et al., 2002; Lynch et al., 2012; Yang, 2003). Especially the interaction of clade A PP2Cs with ABF transcription factors of the bZip family provides a direct link in the control of ABA-dependent gene regulation. It is known that under stress conditions, the phosphorylation of ABF TFs by triggers transcriptional activation of target genes (Kobayashi et al., 2005). According to Lynch et al. (2012), clade A PP2Cs interact with ABFs in yeast and co-localize with them also in plants. Based on these findings, such a direct physical interplay of clade A PP2Cs with ABFs was investigated. In preliminary Y2H analyses, binary interactions of clade A PP2Cs with ABI5 could be confirmed (Fig. 36).

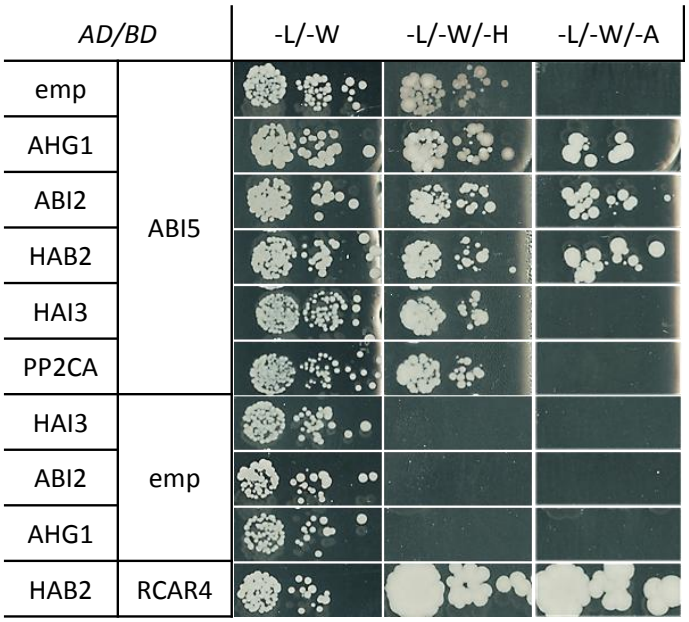


Fig. 36: Y2H interaction of clade A PP2Cs and ABI5. Combinations of five clade A PP2Cs were tested against ABI5 TF for interaction by spotting on selective media. Growth on -L/-W reflects positive dual transformation and growth on triple selective media (-L/-W/-H or -L/-W/-A) indicates interaction. Empty vector (emp) and HAB2/RCAR4 combinations serve as negative and positive controls, respectively.

The preliminary Y2H experiments presented in Fig. 36, confirm a direct, physical connection of PP2Cs and ABFs. Using the His-Autotrophy reporter, no distinct interactions could be detected due to leaky empty vector (emp) controls. However, HAB2, AHG1 and ABI2 clearly interact with ABI5 using the Ade-Autotrophy reporter system, which is more selective in detecting interactions. These binary interactions represent a physical link of PP2C function to transcriptional regulation.

2.4 PP2C-RCAR-like signaling modules

The co-receptor function of RCARs and PP2Cs is confirmed by various biochemical and *in vivo* experiments (Antoni et al., 2012; Nishimura et al., 2010; Yin et al., 2009). The mode of action, i.e. ligand-dependent regulation of signaling modules, might be conserved for other Bet v 1 superfamily members. MLPs are the proteins most closely related to the RCARs in Arabidopsis. This heterogeneous group of proteins shares the common helix-grip fold and is known to be involved in the binding and transfer of small molecules (Lytle et al., 2009; Radauer et al., 2008).

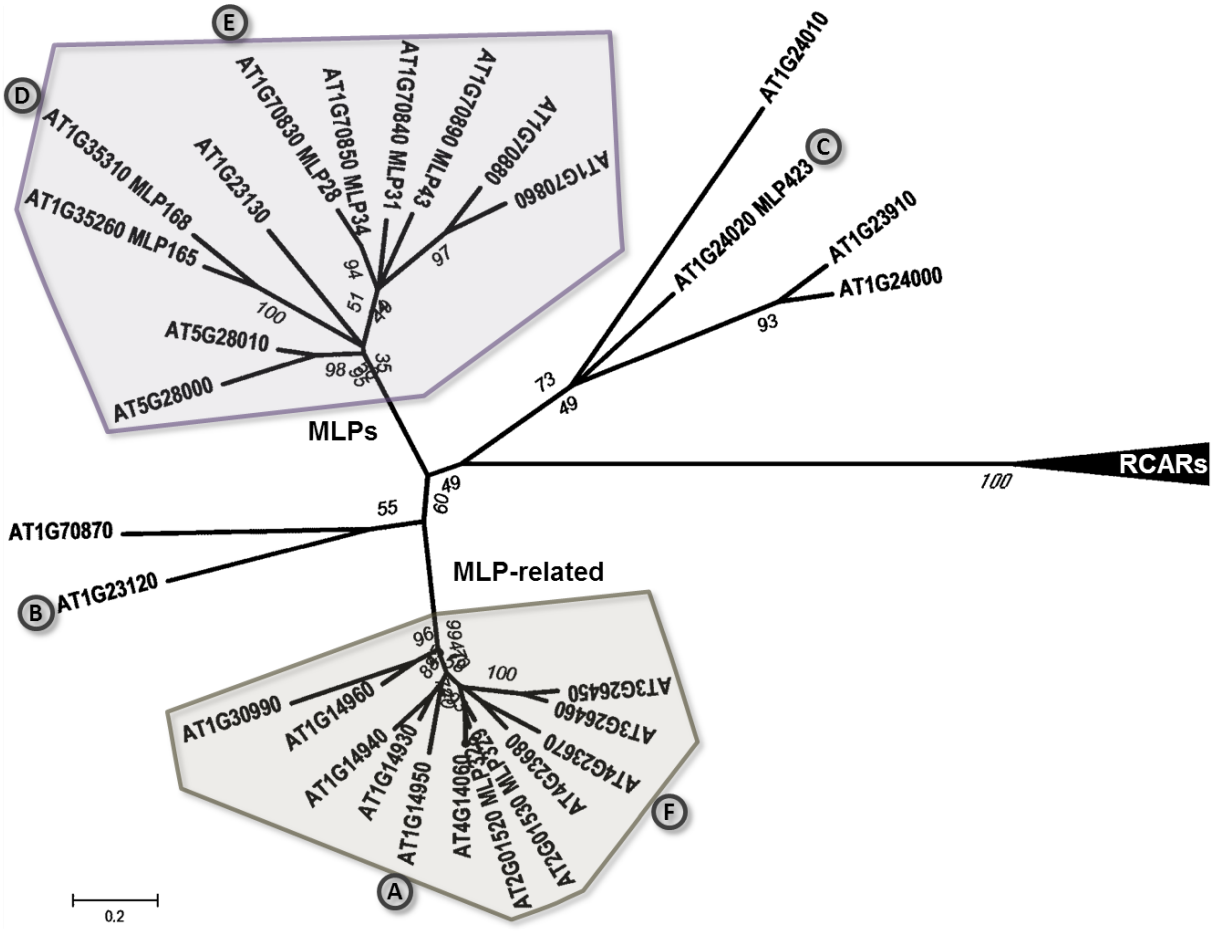


Fig. 37: Phylogenetic analysis of the MLP superfamily of *A. thaliana*. The unrooted Maximum Likelihood consensus tree was corrected using Poisson correction model (Zuckerkandl and Pauling, 1965). Numbers indicate bootstrapped frequencies in 200 replicates (Felsenstein, 1985). Branch lengths represent evolutionary distance and are measured in the number of amino acid substitutions per site. The RCAR subfamily tree is collapsed (see Fig. 5 for detail). Single genes are enumerated and provided with names, where applicable. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The two major subfamilies (MLPs and MLP-related proteins) are highlighted and MLPs selected for subsequent Y2H analyses are labeled (A-F).

For an overview of the phylogenetic relation between RCARs and MLPs is presented as an unrooted evolutionary tree in Fig. 37. The 29 MLP and MLP related genes were identified using BLAST homology (Altschul et al., 1990) online tools (available at http://blast.ncbi.nlm.nih.gov/Blast.cgi) on the RCAR proteins. The CDS of the corresponding genes was retrieved from TAIR database (Huala et al., 2001) and processed for further analyses. Using MEGA5 (Tamura et al., 2011) a consensus tree was calculated for the MLP family (Fig. 37). The RCAR subfamily is included as collapsed branch (compare Fig. 5). The 29 family members form two major clusters of closely related genes, which are highlighted and grouped as MLPs and MLP-related proteins. For the following Y2H interaction screen six genes were randomly chosen and labeled A-F.

2.4.1 PP2C-MLP interaction screen

To test for conservation of a signaling relay similar to PP2C-RCAR complexes, a screen for Y2H interaction analyses was set up. The six randomly selected MLPs (A-F) plus two RCARs (RCAR1 and RCAR6) were cloned in pBridge BD vectors. Additionally, 21 PP2Cs randomly chosen from the different phosphatase subfamilies (labeled #1-21) plus ABI1, ABI2, HAB1 and HAB2 were cloned and tested in pGAD AD vectors (for the full list of the genes tested please refer to Table 3 and Table 4). Firstly, binary interaction of the PP2Cs with the different MLPs was tested by spotting the combinations as dilution series on selective media. Growth on double selection medium (-L/-W) reflects positive dual transformation of pGAD and pBridge vectors. Yeast proliferation on triple selection (-L/-W/-H) indicates protein-protein interaction of the candidate proteins. Promising candidates should then be selected for further analyses in β-Gal assays to quantify the extent of interaction.

It is known for certain combinations of clade A PP2Cs and subfamily III RCARs that Y2H interaction requires ABA for complex stabilization and its detection in the Y2H system. A similar situation might occur for PP2C-interacting MLPs and probable unknown ligands. Therefore, additional selective plates were supplemented with a mixture of major phytohormones (HM) or a plant cell extract (PE). The HM contains natural and synthetic plant hormones of the different classes (e.g. IAA and 2,4-D auxins, kinetin and BAP cytokinins) as well as important low molecular weight signaling molecules (see Table 2 for detailed composition). The PE was prepared from plant suspension cell culture and contained hydrophobic substances of low molecular weight purified on C_{18} columns (see 4.2.13 for preparation). HM in the final concentrations indicated and PE [1:50] were supplemented to triple selective plates selecting for interaction (-L/-W/-H).

Table 2: List of phytohormones supplemented in Y2H selection plates. Substance classes: a - auxin, c - cytokinin, e - ethylene, g - gibberellin, j - jasmonate, s - salicylate, o - others

Substance	Final concentration [µM]	class	Full name / remarks
IAA	50	a	Indole-3-acetic acid
2,4-D	50	a	2,4-Dichlorophenoxyacetic acid
Thidiazuron	50	c	Phenylurea cytokinin
Kinetin	10	c	Adenine cytokinin
BAP	10	c	6-Benzylaminopurine
2iP	10	c	6-(γ,γ-Dimethylallylamino)-purine
ACC	1	e	1-Aminocyclopropane-1-carboxylic acid
GA3	10	g	Gibberellic acid
MeJA	50	j	Methyl-jasmonic acid
SA	50	s	Salicylic acid
Acetosyringone	50	o	3,5-Dimethoxy-4-hydroxyacetophenon
Cinnamic acid	50	o	

The identification of regulatory modules analogous to the PP2C-RCAR relay might foster our understanding of vital plant signaling pathways. In this Y2H approach, a representative compilation of PP2C-MLP interactions was analyzed. The combinations of 8 MLP-like proteins (6 MLPs and 2 RCARs) with 25 PP2Cs (21 random PP2Cs plus four ABI1-like PP2Cs) resulted in 200 possible PP2C-MLP complexes for targeted interaction screening in yeast. AD plasmid containing the PP2Cs and BD plasmids with MLPs were sequentially transformed into AH109 Y2H strain. The proteins selected for the interaction screen are listed in Table 3 and Table 4.

Table 3: List of MLPs tested in Y2H interaction screen.

Abbreviation	Gene	Name/description
MLP A	At1g14950	Bet v 1 lipid transport superfamily protein
MLP B	At1g23120	Bet v 1 lipid transport superfamily protein
MLP C	At1g24020	MLP423
MLP D	At1g35310	MLP168
MLP E	At1g70830	MLP28
MLP F	At4g23670	Bet v 1 lipid transport superfamily protein
RCAR1	At1g01360	RCAR1/PYL9
RCAR6	At5g45870	RCAR6/PYL12

Table 4: List of PP2Cs tested in Y2H interaction screen. Clade A PP2Cs are highlighted in grey.

Abbreviation	Gene	Name/description	Clade
ABI1	At4g26080	ABI1	A
ABI2	At5g57050	ABI2	A
HAB1	At1g72770	HAB1	A
HAB2	At1g17550	HAB2	A
PP2C #1	At3g11410	PP2CA/AHG3	A
PP2C #2	At5g59220	HAI1	A
PP2C #3	At1g07430	HAI2/AIP1	A
PP2C #4	At2g29380	HAI3	A
PP2C #5	At1g47380	PP2C	H
PP2C #6	At1g78200	PP2C	F
PP2C #7	At2g25070	PP2C	I
PP2C #8	At5g02760	PP2C	D
PP2C #9	At3g16560	PP2C	C
PP2C #10	At1g48040	PP2C	G
PP2C #11	At2g20050	PP2C	L
PP2C #12	At5g27930	PP2C	E
PP2C #13	At2g40180	PP2C5	B
PP2C #14	At5g66720	PP2C	K
PP2C #15	At5g66080	PP2C	D
PP2C #16	At4g31750	WIN2	F
PP2C #17	At2g33700	PP2CG1	G
PP2C #18	At1g79630	PP2C	E
PP2C #19	At5g51760	AHG1	A
PP2C #20	At1g07160	PP2C	B
PP2C #21	At1g07630	PLL5	C
PP2C #22	At1g16220	PP2C	E

The PP2Cs were selected from eleven different subfamilies (see Fig. 3 for phylogeny). Binary interactions were determined for all combinations by dilution series spotting. Growth on double selective media indicates positive dual transformation of AD and BD vectors and growth on triple selection indicates positive interaction. Empty pBridge and pGAD combinations were transformed as controls to detect auto-activation of AD and BD constructs, respectively. The combinations analyzed were also spotted on HM and PE supplemented triple selection media to test for ligand-dependent interactions. Examples of these interaction analyses for His-autotrophy of PP2C-MLP combinations are depicted in Fig. 38.

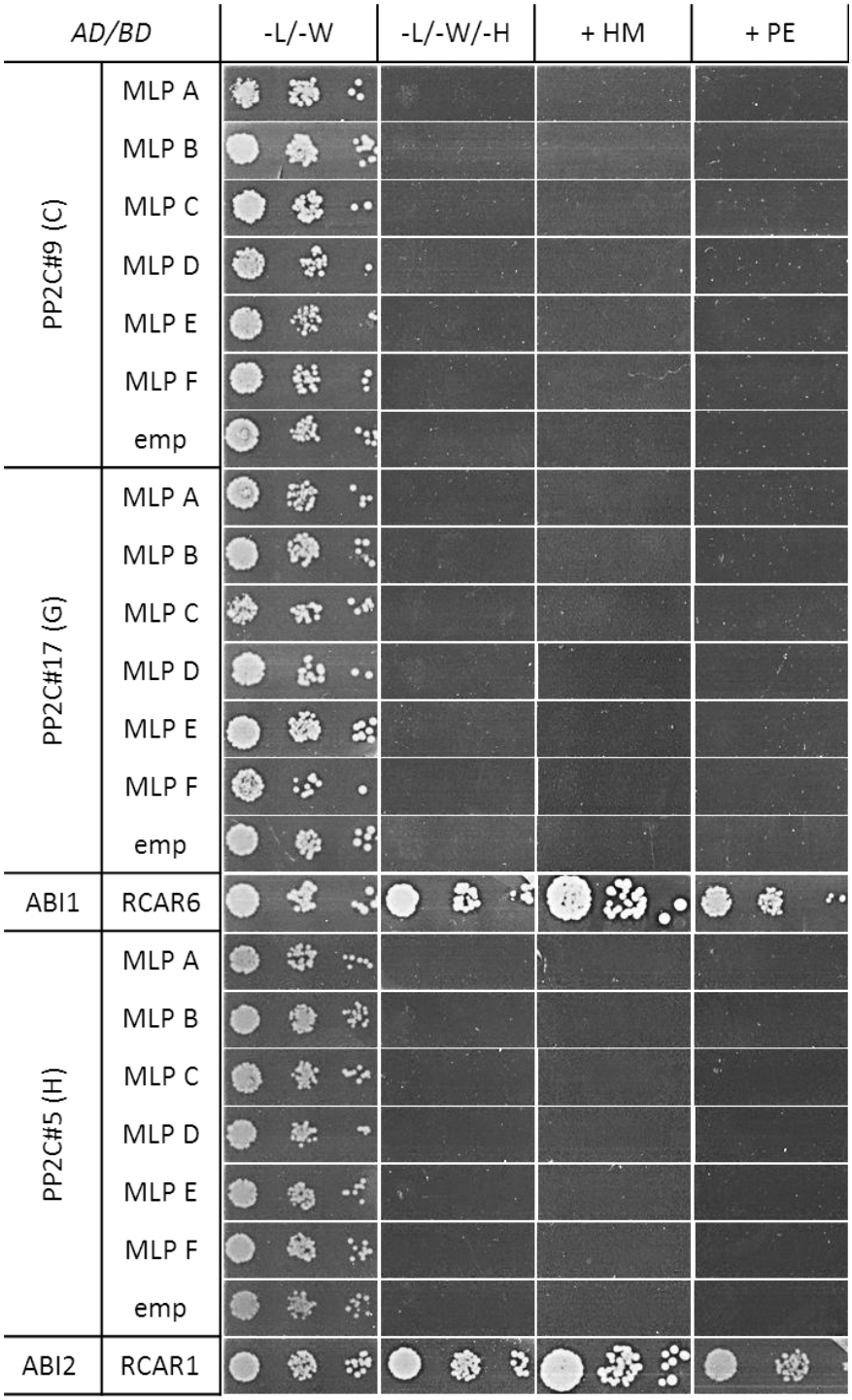


Fig. 38: Interaction of PP2Cs and MLPs in the Y2H-system. Exemplary combinations of PP2C#9, PP2C#17 and PP2C#5 with MLPs (A-F) are spotted as dilution series. His-autotrophic growth on triple selective media (-L/-W/-H) was used as a read-out for interaction. Ligand-dependency was tested in triple selective media including a hormone mixture (HM) or plant cell extract (PE). ABI1/RCAR6 and ABI2/RCAR3 serve as positive, empty vector (emp) combinations as negative controls, respectively.

The extensive Y2H analyses revealed interaction exclusively for clade A phosphatases with RCAR proteins (Fig. 38). The four PP2Cs ABI1, ABI2, HAB1 and HAB2 from clade A were found to constitutively interact with both RCAR1 and RCAR6 in the heterologous yeast system. This interaction

was independent of the ligand ABA (compare Fig. 13). Furthermore, three of the additional clade A PP2Cs included in the assay showed interaction with RCAR1 and RCAR6, namely HAI2, HAI3 and PP2CA (data not shown). For the PP2Cs from the other clades no interaction with RCARs could be detected. Additionally, none of the MLPs tested against any of the different PP2Cs showed growth on the respective selection media. Yeast lines carrying PP2C#5, PP2C#9 and PP2C#17 (from clade H, C and G, respectively) in combination with the six MLPs (A-F) exemplify the His-autotrophy interaction analyses (Fig. 38). The empty BD vector (emp) combinations of the PP2Cs were clearly negative and showed no background activation of the reporter. Empty AD vector combinations of MLPs and RCARs consonantly did not exhibit growth (data not shown). Also no proliferation was observed on HM and PE plates for PP2C-MLP combinations other than clade A phosphatases with the two RCARs tested. A deleterious effect of the hormones or the plant cell extract could be excluded because PP2C-RCAR positive controls were not inhibited in growth compared to normal selection media (-L/-W/-H). To affirm the results obtained from the His reporter system, a representative selection of yeast lines was studied in standard β-Gal activity assays.

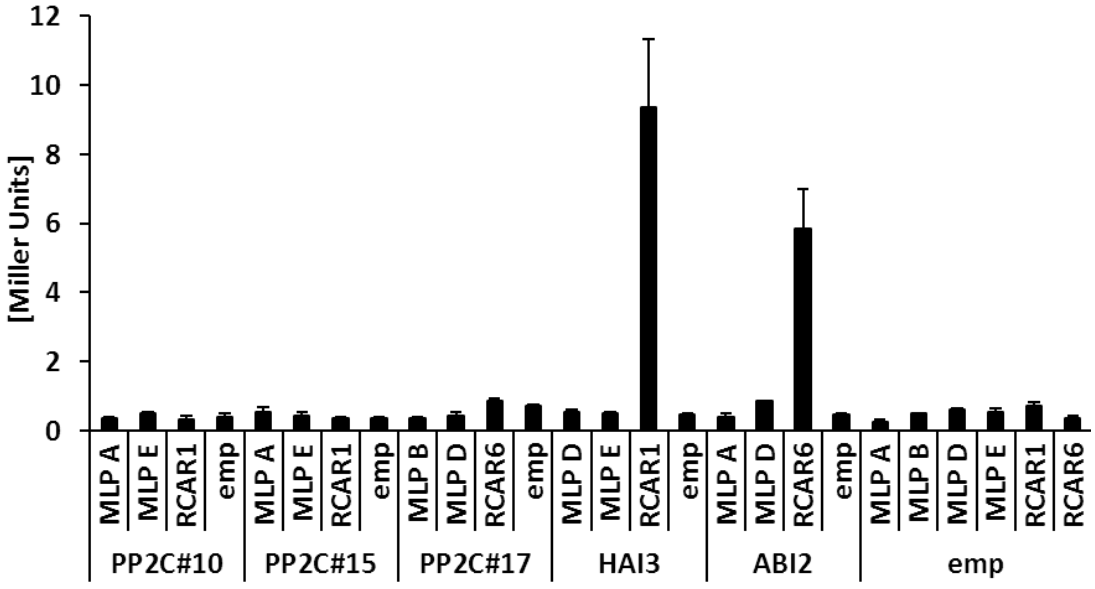


Fig. 39: Quantitation of PP2C-MLP interaction in Y2H. Exemplary combinations of PP2C#10, PP2C#15, PP2C#17, HAI3 and ABI2 with different MLPs or RCARs were assayed for interaction. β-Gal activity serves as read-out for interaction. Values are means (± SD) of three independent transformant lines and given in Miller units. HAI3/RCAR1 and ABI2/RCAR6 combinations serve as positive, empty vector (emp) combinations as negative controls. Values ≤1 indicate absence of interaction.

The use of the β-Gal reporter system confirmed the results of the His-autotrophy screen for PP2C-MLP interactions (Fig. 39). In these quantitative assays again only PP2Cs from clade A in combination with RCAR1 and RCAR6 showed significant activation of the reporter. HAI3 interaction with RCAR1

with ~ 9 Miller units was approximately 1,5 times stronger than ABI2-RCAR6 interaction in this assay. These results are perfectly consistent with the interaction data gathered for HAI3 and ABI2 in combination with subgroup I and subgroup II in previous assays (compare Fig. 12). The other PP2Cs tested (PPC2#10, PP2C#15 and PP2C#17) did not show interaction with any of the MLPs tested. With β-Gal activities below 1 Miller unit these experiments confirmed the results from the His-autotrophy reporter system.

The aforementioned interaction studies might be restricted due to the absence of a supposed ligand, which could be necessary to stabilize MLP-PP2C coupling. Nevertheless, a coincidence of MLPs and PP2Cs in distinct defense related signaling cascades is apparent (Golem and Culver, 2003; Lee et al., 2008). For example, MLP34 (At1g70850) and a clade F PP2C (At4g31750) were independently identified in a screen for genes affecting parthenocarpy. Subsequent analyses revealed that both genes play a role in auxin- and defense-related traits (A. Spena, personal communication). The same PP2C had been identified before in a screen for plant proteins interacting with the bacterial effector protein HopW1-1 from pathogenic *Pseudomonas syringae* and it was termed HopW1-1 interacting protein 2 (WIN2) (Lee et al., 2008). WIN2 was shown to be an important regulator of SA-mediated resistance. It preferentially interacted in yeast with an n-terminally truncated fragment of the bacterial effector protein (tHopW1-1). MLP34 and its closely related homolog MLP28 are unique within the MLP superfamily because both full length proteins consist of an intra-molecular tandem repeat of two Bet v 1 helix-grip fold structures. According to the specifications of A. Spena regarding the fragments identified in the original screen, a c-terminally truncated version of MLP34 (tMLP34) was generated. To confirm a possible direct physical interaction of these two proteins, targeted Y2H analyses were performed.

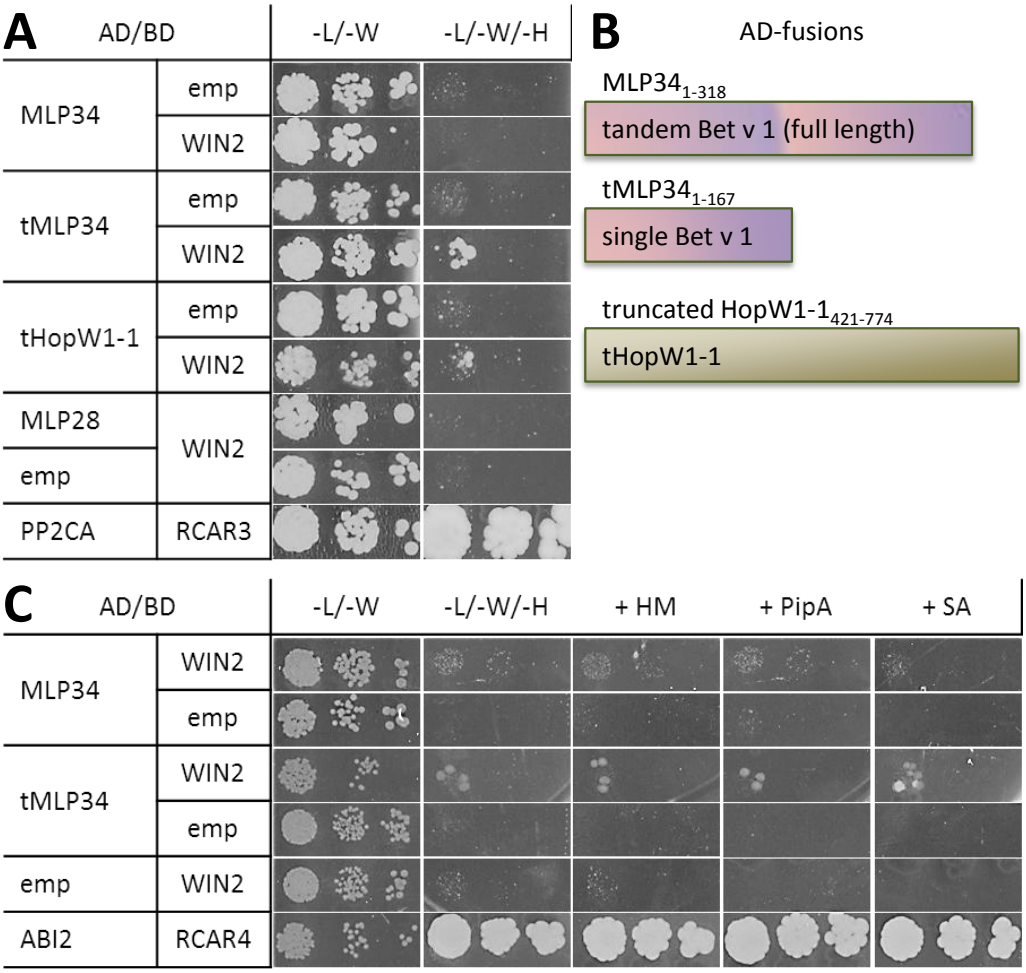


Fig. 40: Interaction of MLP34 and WIN2. (A) The Y2H interaction of MLP34 and WIN2 was determined by spotting a serial dilutions on selective media (-L/-W/-H). WIN2 was tested against full length MLP34, truncated MLP34 (tMLP34) and closely related MLP28. A truncated version of bacterial effector tHopw1-1 in combination with WIN2 and PP2CA/RCAR3 were used as positive controls. Empty vector (emp) combinations serve as negative control. Plates were incubated for 12 d. (B) The scheme depicts the different versions of interactors tested with WIN2. (C) A ligand-dependent stabilization was tested by adding a phytohormone mixture (HM), pipecolic acid (PipA) or SA to the selection media (-L/-W/-H). Plates were incubated for 8 d and ABI2/RCAR4 and emp/WIN2 combinations serve as positive and negative controls, respectively.

Fig. 40 shows the interaction of MLP34 and WIN2 in the heterologous yeast system. The full length MLP34 did not interact with WIN2 PP2C. Instead, there was a weak interaction of WIN2 and the truncated version of MLP34 (tMLP34). In the tMLP34 construct the c-terminal tandem unit is removed (Fig. 40B). This modification allows interaction with WIN2. The coupling was weak but seems to be independent of a ligand. A similar interaction of WIN2 was reported for the bacterial effector protein HopW1-1 (Lee et al., 2008). The truncated version of the effector (tHopW1-1) was included as a control in these assays and it confirmed a weak interaction of tHopW1-1 and WIN2 (Fig. 40A). Up to now, this is the first evidence of a PP2C-MLP interaction resembling the PP2C-RCAR docking. In an attempt to stabilize PP2C-MLP binding by providing a possible ligand, the selective

media (-L/-W/-H) were supplied with a mixture of phytohormones (+ HM, see Table 2 for composition), plant defense response-mediating pipecolic acid (PipA) (Navarova et al., 2012) or SA. The addition of these substances did not promote interaction of the candidate proteins resulting in increased proliferation of the Y2H clones.

2.4.2 Transcriptional regulation of RCAR-related MLPs

With respect to the transcriptomic changes of clade A PP2Cs and RCARs in the course of stress response and throughout development, the expression patterns of MLPs or WIN2-related PP2Cs may also reflect their physiological function. Transcriptional profiles, similar to the ones presented for the clade A PP2Cs (see Fig. 18, Fig. 19, Fig. 20 and Fig. 21), were created for the MLP family members. After analyses of the datasets available for 27 out of the 29 MLP proteins from GenExpress (Schmid et al., 2005; Toufighi et al., 2005), the regulation of MLPs at the transcriptional level turned out to be heterogeneous. Transcript levels were visualized throughout different developmental time-points or under different environmental conditions using the eNorthern tool available at the BAR (Kilian et al., 2007; Toufighi et al., 2005). Exemplary for these analyses, MLP transcript abundance and alterations during seed maturation and germination are presented in Fig. 41.

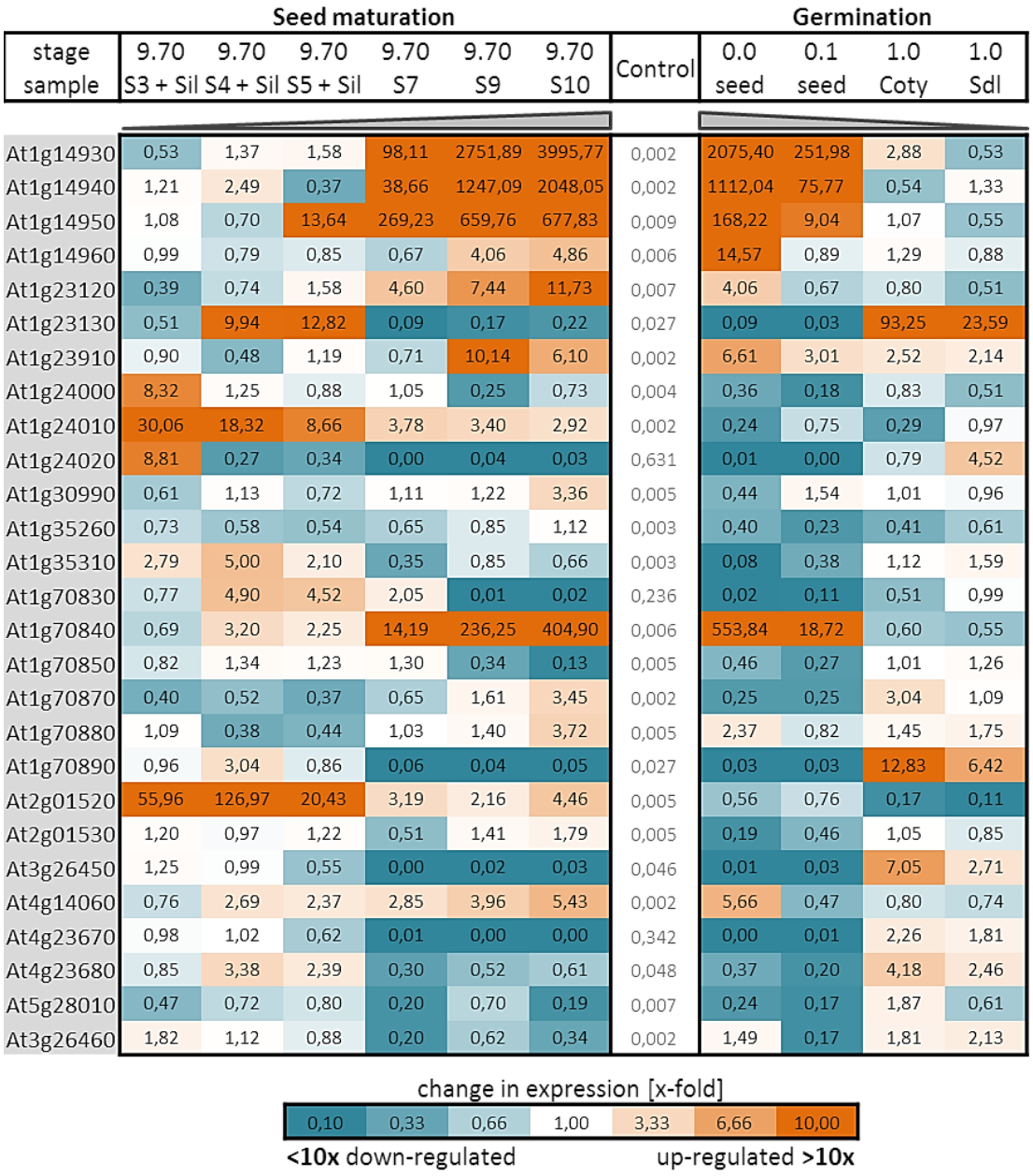


Fig. 41: Transcriptional regulation of MLPs in seed development and germination. The expression of the MLP genes is shown at different time points relative to the developmental baseline expression median of the respective gene. Transcript abundance relative to ubiquitously expressed reference gene UBQ10 is indicated by the ratio given in the control column. Developmental stages and samples are indicated on top, according to (Boyes et al., 2001; Kilian et al., 2007; Schmid et al., 2005). Intensities of blue and orange indicate down- and up-regulation, respectively. Grey slopes symbolize increasing seed maturity and decreasing seed dormancy status, respectively.

The MLP superfamily is variably regulated at the transcriptional level throughout seed development and germination (Fig. 41). The general expression levels of these RCAR-related genes compared to UBQ10 constitutively expressed reference gene are very low (ratios in Control column). Only At1g24020, At1g70830 and At4g23670 exhibit higher levels of expression at a ratio of > 0,1

compared to UBQ10 reference. The relative expression patterns are variable within the MLP superfamily. Especially in seed maturation and germination and subsequent seedling establishment, the transcription levels of different MLP genes are divergent (Fig. 41). Comparison of the expression patterns of MLPs displays strong up-regulation of At1g14930, At1g14940, At1g14950 and At1g70840 in seed maturation. In the same stages At1g24020, At1g70830, At1g70890, At3g26450 and At4g23670 are markedly repressed. There is no consistent transcriptional response of MLPs similar to the PP2C-RCAR inverse regulation in ABA-dependent processes. Also after stress or hormone treatment a distinct pattern MLP regulation remains elusive (data not shown). The alterations in transcript abundance during pathogen response reported by previous studies (Devos et al., 2006; Siemens et al., 2006) were not reflected in the available datasets. The same holds true for the clade F PP2Cs. Unlike the ABI1-like phosphatases in ABA signaling, the involvement of WIN2 and PAPP2C in defense signaling is not reflected by the corresponding expression patterns (data not shown). Hence it is not possible to identify a conserved mode of PP2C-MLP regulation of SA signaling at the transcriptional level.

2.4.3 Regulation of PP2C activity by MLPs

For further investigation on the function and regulation of the identified complex, the phosphatase activity of recombinant WIN2 was to be assayed *in vitro.* Expression constructs of WIN2, MLP34, tMLP34 were generated in pQE70 vectors for heterologous expression in M15 pREP4 *E. coli* cells. Culture conditions and required levels of isopropyl-β-D-thiogalactopyranosid (IPTG) were optimized by testing different induction times (0/30/60/120 or 240 min) at different IPTG concentrations (0/0,1/0,25/0,5 or 1 mM) (data not shown). The bacteria were grown up to an OD_{600} of ~ 0,5 before inducing protein expression of the desired protein by IPTG application. Optimal protein yields for tMLP34 were achieved at 0,5 mM IPTG for 3 h. WIN2 was induced for 3 h at 1 mM IPTG. After lysis the soluble protein fraction was purified using Ni-TED 2000 columns (Protino Kit, Macherey-Nagel) and hexahistidine (6xHis)-tagged recombinant protein could be recovered. (see 4.2.14 for procedure). The calculated molecular weight of the tagged recombinant proteins (34 kDa for WIN2 and 18,7 kDa for tMLP34) were consistent with the proteins sizes determined by SDS-PAGE and after Western Blotting (data not shown).

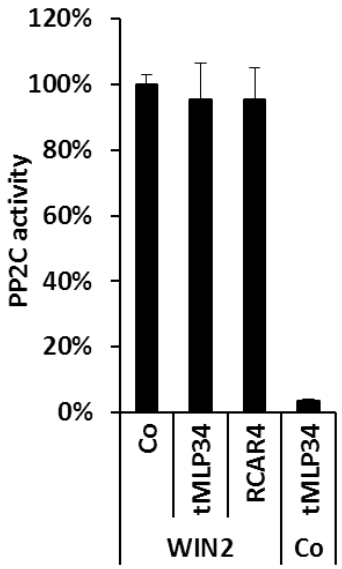


Fig. 42: *In vitro* phosphatase activity of WIN2. The relative catalytic activity of WIN2 PP2C on MUP-substrate was measured in presence of buffer alone (Co), the truncated MLP34 (tMLP34) or RCAR4 control. tMLP34 alone serves as negative control.

The phosphatase activity of recombinant WIN2 on methylumbelliferyl phosphate (MUP) fluorogenic substrate was assayed *in vitro* (Fig. 42). Therefore, recombinantly expressed PP2C and tMLP34 proteins were purified and their concentrations were determined after SDS-PAGE. For the assays approximately 250 ng of each protein were used per measurement. The measured activity of the PP2C alone, 669650 ± 18561 RFU/sec, was set as 100 %. An addition of tMLP34 to the PP2C did not significantly change phosphatase activity towards the artificial substrate. In control reactions with the PP2C and RCAR4 in equal amounts the phosphatase activity also remained unchanged. tMLP34 protein alone did not possess phosphatase activity. These experiments confirmed the catalytic activity of the PP2C WIN2 reported by Lee et al. (2008). Addition of recombinant tMLP34, which showed interaction in yeast, did not significantly alter phosphatase activity. Analogous *in vitro* experiments with RCARs and corresponding clade A PP2Cs also did also not show a significant inhibition of the phosphatase in the absence of a ligand (Ma et al., 2009).

2.4.4 MLP function in SA signaling

Based on previous reports, in which clade F PP2Cs as well as MLPs play a role in defense signaling (Osmark et al., 1998; Wang et al., 2012). In an additional approach to study MLP and PP2C function, a new protoplast reporter was constructed to assess biotic stress-related responses. Plant defense signaling is reflected by a strong induction of pathogenesis-related (PR) proteins largely mediated by SA (Cameron et al., 1999; Hong et al., 2005). In the new reporter construct the 1,2 kb promoter

region upstream of the PR1 protein amplified from Col wt genomic DNA was placed in front of a LUC reporter gene. Therefore, the reporter construct commonly used at the Institute for analyses of ABA-dependent signal transduction was modified (#3041 of the Institute´s strain collection). The pRD29B fragment in this plasmid was removed and replaced with the promoter region of PR1 (pPR1). Similar constructs were used earlier to visualize SA-mediated pathogen response *in planta* (Hermann, 2009; Hong et al., 2005; Pape et al., 2010).

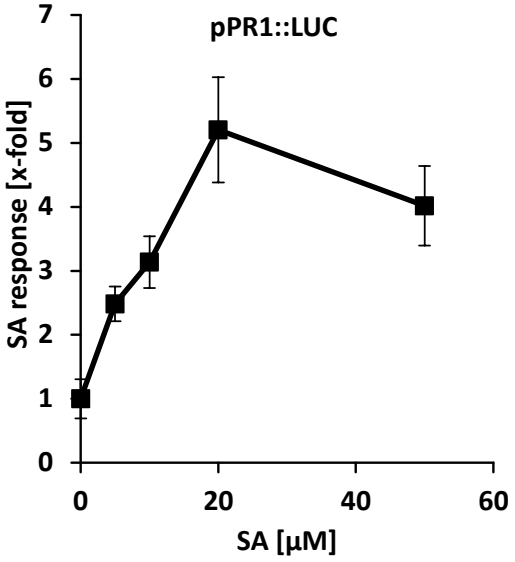


Fig. 43: pPR1 reporter system in protoplasts. The SA concentration-dependent activation of the pPR1::LUC reporter is shown as x-fold induction of SA response relative to an untreated control. Samples were incubated for 18 h. The mean GUS activity of these samples is 0,140 (± 0,063) RFU/sec.

The SA response of the newly established reporter system is shown in Fig. 43. The pPR1::LUC reporter showed reproducible SA-dependent induction in Col wt protoplasts. With increasing SA concentrations the LUC activity increased relative to the co-transfected 35S GUS constitutive reference used for normalization. Maximal induction was observed at 20 µM SA. However, detailed investigation of the relative florescence units (RFU) values produced by measuring the GUS internal standard revealed a drop of constitutive GUS expression levels at higher ligand concentrations above 10 µM SA (data not shown). This finding reflects a decreased viability of the protoplasts. Taking this into account, the following analyses on SA response signaling were performed at 5 µM SA.

The function of MLPs and PP2Cs in defense-related signal transduction was assessed using the new reporter in the protoplast system. Therefore, protoplast expression vectors of all six MLPs tested in

the Y2H screen (MLP A - F) were generated by transferring the CDS fragments from the yeast vectors into compatible pSASF vectors (see 4.2.12). Seven protoplast constructs were generated for five additional MLPs and two truncated versions of MLP28 and MLP34. Among the additionally cloned MLPs there was MLP31, which is closely related to MLP28/34 but does not possess their tandem repeat double Bet v 1 structure. Furthermore, constructs of four supposedly defense-related PP2Cs were included in these protoplast assays. A list of the genes included in these analyses is shown in Table 5.

Table 5: List of genes tested in SA-response assays in protoplasts.

Abbreviation	Gene	Name/description
MLP G	At2g01520	ZCE1 / MLP328
MLP H	At5g28000	Bet v 1 lipid transport superfamily protein
MLP I	At1g23910	Bet v 1 lipid transport superfamily protein
MLP31	At1g70840	MLP-like protein 31
MLP34	At1g70850	full length MLP34
tMLP34	At1g70850	truncated MLP34
tMLP28	At1g70830	truncated MLP28
PP2C#6	At1g78200	PP2C (clade F)
PP2C#12	At5g27930	PP2C (clade E)
PP2C#18	At1g79630	PP2C (clade E)
PP2C#16	At4g31750	WIN2 (clade F)

The effects of ectopic expression of these components in protoplasts could be analyzed using the new reporter construct pPR1::LUC. The truncated version of MLP28 and MLP34 consist of the N-terminal Bet v 1 helix grip fold single unit ($MLP28_{1-173}$ and $MLP34_{1-167}$). Based on their differential behavior in the yeast assays these short versions were also tested in protoplasts for their effect on SA signal transduction.

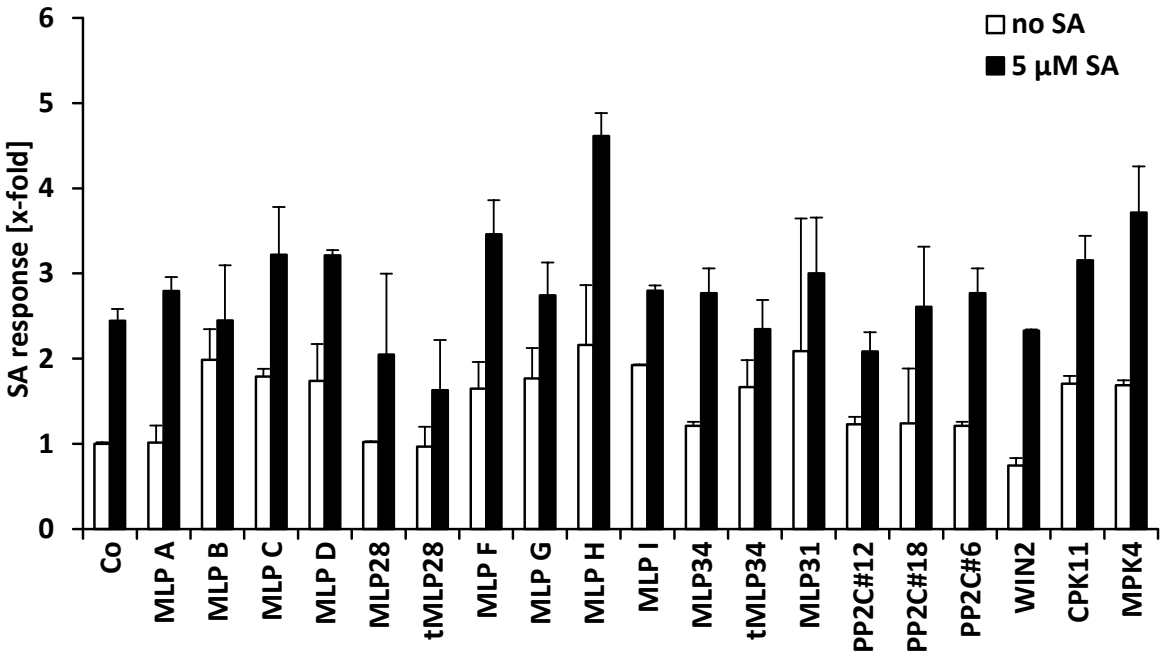


Fig. 44: Effect of MLPs and PP2Cs on SA response. The effect of co-expression of different MLPs, PP2Cs or two defense signaling-related kinases (CPK11 and MPK4) is shown relative to untreated empty vector control (Co) in the absence or presence of 5 µM SA. Samples were incubated for 18 h and for each effector construct 1 µg of plasmid DNA was transfected. Mean GUS activities of the samples in this experiment are 0,401 (± 0,124) RFU/sec.

The effects of ectopic expression of different MLPs and PP2Cs are depicted in Fig. 44. Most of the MLPs expressed, including truncated versions of MLP28 and MLP34 (tMLP28 and tMLP34, respectively) did not significantly perturb pPR1::LUC reporter activation. A slight, approximately 1,5-fold increase in SA response was mediated by MLPs C, D, F and H. Due to the low general induction of the reporter by SA and the high standard deviation between the replicates, this change may not be significant. The same holds true for the PP2Cs from clade E (PP2C#12 and PP2C#18) or clade F (PP2C#6 and WIN2). Over-expression of the phosphatases did not significantly alter SA-dependent reporter activation. For example, WIN2 expression reduced SA response to ~75 % of the empty vector control (Co) without exogenous SA. CPK11 and MPK4 were included in the analyses as they are known to be positively regulating defense signaling (Boudsocq et al., 2010; Brodersen et al., 2006). Their co-expression slightly induced pPR1 signaling in the system presented here. An induction similar to the one induced by CPK11 and MPK4 co-expression was seen for MLP C, D, F and H, particularly in the absence of exogenous SA. MLP-dependent induction of SA response without exogenous SA was also observed for MLP B and MLP 31. Taken together, the present protoplast studies give no clear indication for a direct effect of MLPs or PP2Cs on defense signaling.

3 DISCUSSION

3.1 Combinatorial interaction of ABA receptor complexes

The discovery of the soluble ABA co-receptor complex, consisting of RCAR sensory proteins and the PP2C master signaling regulators, was a key for understanding the regulation of ABA-dependent processes (Ma et al., 2009; Park et al., 2009). First results of yeast heterologous interaction studies conducted in the present work were highly consistent with previously reported interactions of ABI1 and ABI2 with RCAR1 and RCAR3 (Ma et al., 2009; Szostkiewicz et al., 2010; Yang, 2003), as well as of HAB1 with 13 out of the 14 RCARs (Park et al., 2009). Thus, the heterologous interaction analysis in yeast appears to be an appropriate system to study the combinatorial assembly of all the possible co-receptor complexes.

The compilation of 9 PP2C x 14 RCAR interactions tested in the present work, constitute the first systematic analysis of the combinatorial assembly of all conceivable co-receptor complexes.

PP2C	RCAR subfamily I	RCAR subfamily II	RCAR subfamily III
ABI1	+	+ (A)	A
ABI2	+	+ (A)	A
HAB1	+	+ (–/A)	A
HAB2	+	+ (A)	A
PP2CA	+	+	A
HAI3	+	+ (–/A)	– (A)
HAI2	+	+ (–/A)	– (A)
HAI1	–	–	–
AHG1	– (A)	– (A)	–

Fig. 45: Summary of PP2C-RCAR Y2H interaction. The combinations of clade A PP2Cs with RCAR subfamilies I, II and III detected in yeast, are illustrated as constitutive, absent and ABA-dependent interactions (**+**, **–** and **A**, respectively). Single exceptions for PP2C interaction amongst RCAR subfamilies are indicated by subscript (–/A). Blue or light blue coloring indicates constitutive interaction of all or most subfamily members, respectively. Green and white coloring indicates ABA-dependent interaction or no interaction, respectively.

Fig. 45 summarizes the possible receptor complexes between the nine clade A PP2Cs and the three different RCAR subfamilies identified in yeast. Apart from single exceptions, indicated by subscript symbols, the single members of the RCAR subgroups exhibit similar interaction patterns in combination with the distinct PP2Cs. Altogether, this summary depicts the constitutive and ABA-independent interactions detected for RCAR subfamily I proteins with seven out of all nine PP2Cs from clade A. In a similar fashion, subfamily II RCARs exhibited constitutive interaction with most of the PP2Cs tested. Furthermore, single RCARs showed clear preferences for one or the other PP2C. For example, RCAR9 showed ABA-dependent interaction with HAI2 and HAI3, while it interacted constitutively with the ABI1-like PP2Cs. Interestingly, the RCARs out of subgroup III did not constitutively interact with any of the PP2Cs tested. Exceptional patterns of interaction were also observed for two of the PP2Cs. AHG1 only interacted with a small subset of RCARs in the presence of ABA. HAI1 did not interact with any of the RCARs.

The different Y2H experiments performed in the course of this work produced consistent results, using alternate reporter read-out systems. In the His-autotrophy spotting assays, exemplified by Fig. 9, constitutive binary interactions were observed for RCAR3 from subfamily I with seven PP2Cs from clade A. RCAR7 from subfamily II interacted with 6 out of the 9 PP2Cs. RCAR11, representing subfamily III of the RCARs in these analyses, was dependent on the ligand ABA to stabilize the heteromeric complex with five of the tested PP2Cs. Such a ligand-dependent interaction for subfamily III RCARs was also observed by Park et al. (2009) using HAB1 phosphatase. While ABI1 and closely related ABI2, HAB1 and HAB2 exhibited interaction with RCAR11 in the presence of ABA, there was no detectable binding of HAI1, HAI2, or HAI3 to this particular ABA sensor. This clearly indicates interaction preferences, not only among RCARs but also among the PP2Cs.

The ligand-dependent establishment of certain co-receptor combinations could also be visualized by liquid culture Y2H growth assays (compare Fig. 10). Constitutively interacting HAB1-RCAR4 lines proliferated similarly in triple selective media without the ligand and under double selection. Combinations with RCARs from subfamily III did not grow, unless ABA was exogenously supplied to promote stable formation of the complex. These experiments proved that different combinations of RCARs and PP2Cs have different affinities for binding each other and also for binding the ligand. The effect of the exogenously supplied ligand is critical for the formation of the complex. The lag in yeast proliferation, comparing constitutively HAB1-interacting RCAR4 and ABA-stabilized RCAR14 coupling, could be due to low affinity complex formation and the time necessary for the uptake of ABA into the yeast cells (Fig. 11). Combinations of subfamily III RCARs exhibited growth similar to empty vector

controls. This shows that ABA is necessary to induce stable complex formation for certain combinations of PP2Cs and RCARs.

The β-Gal reporter system allows a quantitation of interaction strengths (Gietz et al., 1997). ABI1 and ABI2, for example, show ABA-independent interaction with RCAR3 and RCAR7, while RCAR11 interaction is ABA-dependent. A constitutive interaction of HAI group phosphatases HAI2 and HAI3 was observed for RCAR3 and RCAR7 but not for RCAR11. This again reflects preferential and ligand-independent interaction of subfamily I and II RCARs. Surprisingly, the interaction of HAI3 with RCAR3 yielded a very strong signal (around 10 Miller units), which is approximately the double of the value observed for the closely related HAI2 PP2C in combination with the same RCAR. A clear interaction-stabilizing effect of ABA was observed in the combinations of ABI1 and ABI2 PP2Cs with RCAR11. These quantitative results are highly consistent with the data retrieved from the His-growth assays and illustrate the preferential formation of certain co-receptor complexes.

Summing up, the heterologous Y2H studies on the total of 126 possible combinations identified 93 positive interactions. Of those, 61 were ABA-independent and 32 were ABA-dependent interactions. For a subset of 33 tested combinations, no interaction was detected. These analyses are consistant with, and hence confirm, previous studies on the interaction of single PP2C and RCAR family members (Bhaskara et al., 2012; Lee et al., 2012; Park et al., 2009; Peterson et al., 2010). Furthermore, the combinatorial assembly and the number of conceivable ABA co-receptor complexes driving signaling outcome is reminiscent with the formation of auxin co-receptor complexes between 6 TIR1/AFB and 29 AUX/IAA proteins (Calderon Villalobos et al., 2012). However, this is the first report on RCAR7 interaction with clade A PP2Cs. Up to now, only 13 of the 14 RCARs were considered to be PP2C interacting receptor proteins (Fujii et al., 2009; Miyakawa et al., 2012; Park et al., 2009). The assays at hand clearly indicate ABA receptor function for all 14 closely related RCAR proteins.

3.1.1 Structural features of RCAR proteins

The interaction preferences of certain RCARs were reported to be intimately connected to the oligomeric state of these sensory proteins. Subfamily I and subfamily II proteins were postulated to be monomeric enzymes that are freely accessible to the ligand and the PP2C for the formation of the heterotrimeric complex (Dupeux et al., 2011). On the other hand, there are implications that subfamily III RCARs dissociate from a homodimeric state only after ligand-induced conformational changes (Nishimura et al., 2009). Depending on the corresponding affinities for the ligand, for dimerization and for complexation with the PP2C, the RCARs represent a fine-tuned sensory

machinery for ABA perception. These differences might be based on the structural features of the single members and subfamilies.

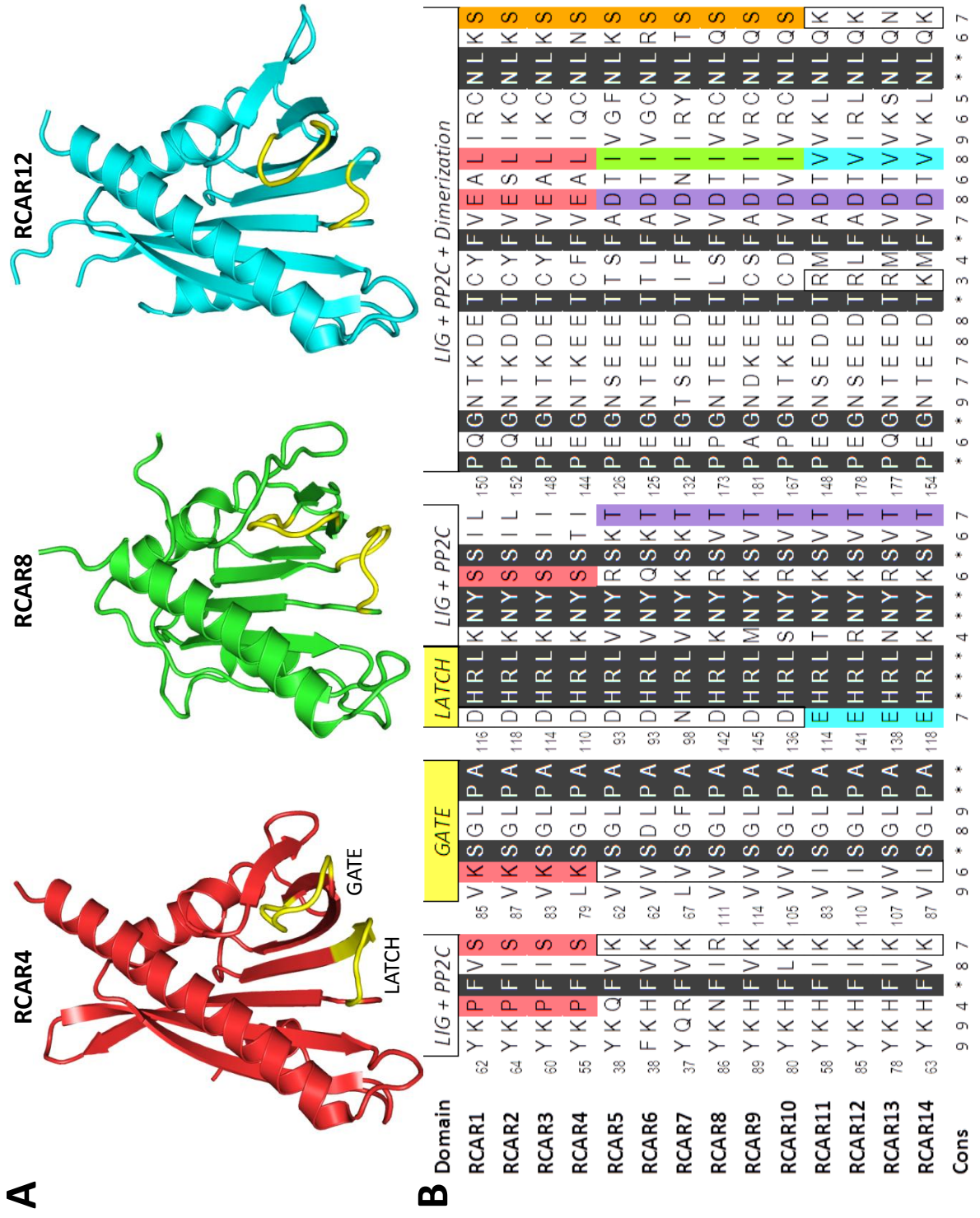


Fig. 46: Structures and alignment of RCAR proteins. (A) The structures of RCAR4, RCAR8 and RCAR12 are shown, based on the PDB datasets 3RT2 (red), 4JDL (green) and 3KAY (cyan), respectively. "Gate" and "Latch" residues are highlighted in yellow. (B) Important RCAR domains involved in ligand binding (LIG), PP2C interaction or dimerization are aligned. Numbers indicate the amino acid position. The bottom line evaluates conservation (Cons) of residues within the protein family from 0-not conserved to *-completely conserved (dark shading). The colors in the alignment correspond to residues conserved in subfamily I (red), II (green), III (cyan) or multiple subfamilies (orange and purple). Boxed residues indicate substantial conservation of critical residues.

The structural characteristics and sequence-specific differences between the RCAR subgroups are illustrated in Fig. 46. The three subfamilies are exemplified by RCAR4, RCAR8 and RCAR12, which are presented as structural models. These are based on PDB datasets and the HHpred structural prediction online toolkit provided by the Max Planck Institute for developmental biology at the University of Tuebingen (Remmert et al., 2012; Soding, 2005; Soding et al., 2005), using "best multiple templates" for RCAR8. The general structure of these three RCAR proteins is highly conserved and comprises the prototypical Bet v 1 helix-grip fold (Iyer et al., 2001). At the structural level, the spatial orientation of vital domains, such as the "gate" and "latch" loops, is strictly conserved in these RCARs (Peterson et al., 2010; Weiner et al., 2010).

Despite the structural similarity, the differences in important RCAR residues have to be responsible for the different binding affinities at the molecular level. In several *in vitro* assays, subfamily I and II RCARs exhibited significantly lower K_d values than subfamily III RCARs for ABA binding (Melcher et al., 2010a; Santiago et al., 2009b; Szostkiewicz et al., 2010). Furthermore, RCAR4 and to a lesser extent also RCAR9 and RCAR10 exhibited ligand-independent phosphatase inhibition also *in vitro* (Hao et al., 2011). At least for RCAR1 or RCAR3, such an ABA-independent inhibition of the PP2Cs was not observed in the assays of using non-peptidic MUP as a PP2C substrate.

At the amino acid sequence level, there are clear distinctions between the subfamilies. Fig. 46B shows an alignment of important amino acids involved in ligand binding (LIG), PP2C interaction (PP2C) and probable dimerization in the RCAR proteins (Hao et al., 2011; Melcher et al., 2009; Miyazono et al., 2009; Santiago et al., 2012; Santiago et al., 2009a). The coloring of residues characteristic for one or two subfamilies illustrates the biochemical differences. Based on the different physico-chemical properties of the amino acids comprised in these important domains, the affinities for ligand binding and PP2C interaction of the RCARs are affected. Mosquna et al. (2011) clearly showed that the mutation of single residues is enough to establish ABA-independent interaction between the PP2C and the RCAR. Their studies identified ten distinct sites within RCAR11, where an exchange of a single amino acid enabled the stable formation of a complex with HAB1 in yeast, even in the absence of the ligand. The combination of these activating mutations potently and selectively stimulated RCAR function *in vitro* and *in vivo*. For instance, RCAR11 point mutations H60P and I84K resemble subfamily I conformation at these particular sites. These modifications promoted ligand-independent interaction with HAB1 PP2C in yeast. Furthermore, the combination of at least three of these mutations, within one engineered RCAR version, markedly increased the inhibition of ABI1, ABI2 and HAB1 phosphatase activity *in vitro*. Of course, the constitutive interaction observed in yeast could be explained by an ABA-independent coupling for certain RCARs. Nevertheless, the

presence of small amounts of ABA or another suitable ligand in yeast, sufficient to induce interaction of the high-affinity receptors from subfamilies I and II with the PP2Cs, cannot be entirely excluded.

3.1.2 Structural features of PP2C proteins

Apart from the structural features of the RCAR proteins, also the PP2C interaction interface contributes extensively to the different binding preferences. The same PP2C residues, which are involved in coordinating RCAR pseudo-substrate interaction, are also involved in the recognition and conversion of cellular substrate, e.g. SnRK2s (Hirayama and Umezawa, 2010; Soon et al., 2012a; Yunta et al., 2011). Moreover, the docking of the downstream target kinases and the regulatory RCARs to the PP2C catalytic site, is based on a conserved grid of important amino acids amongst these different class proteins (Fuchs et al., 2012). Soon et al. (2012a) assessed this overlap as "molecular mimicry". Hence, the specificity of the PP2Cs in coupling to different RCARs might also reflect different binding affinities for other substrate proteins. The structural elements forming the interaction interface and harboring the catalytic site are vastly conserved among the clade A PP2Cs. Nevertheless, N- and C-terminal protrusions are characteristic for single phosphatases and implicate an additional level of regulation (Schweighofer et al., 2004).

As summarized in Fig. 45, also the PP2Cs clearly exhibit preferential binding to distinct RCAR partners. For example, ABI1 and closely related ABI2, HAB1 and HAB2 PP2C featured an overlapping set of interaction partners. Exceptional is only the lack of HAB1-RCAR7 complexation in yeast, which is consistent with the results from Bhaskara et al. (2012). The similar interaction patterns could not only reflect structural homology but also a functional overlap of the ABI1-like subgroup. This assumption is corroborated by the analogous regulation of the PP2Cs on the transcriptional level (compare Fig. 18, Fig. 19, Fig. 20 and Fig. 21). For instance, all three HAI PP2Cs are strongly induced by exogenous ABA and stress (Fujii and Zhu, 2009). However, this transcriptional co-regulation is at least in part reflected by their preferential binding to RCAR proteins. In yeast HAI2 and HAI3 did not interact with subfamily III RCARs, except RCAR14. Bhaskara et al. (2012) tested all three HAI phosphatases against the RCARs in β-Gal activation assays. Their experiments also showed no interaction of the HAI PP2Cs with RCARs from subfamily III. HAI2 and HAI3 strongly interacted with subfamily I RCARs (equal to PYL7-PYL10) in their assays, which is in agreement with the results of the present study. This specific lack of interaction with certain RCARs could be vitally important in RCAR-dependent control of HAI function at the plant level (Bhaskara et al., 2012).

In the heterologous experiments, the interaction of clade A PP2Cs with RCARs was particularly restricted for AHG1 and HAI1. AHG1 is outstanding from the other clade A PP2Cs because of its prominent role during germination (Nishimura et al., 2007) and the discrepancies regarding the conservation of important amino acids (Weiner et al., 2010). Moreover, AHG1 was reported to be not regulated by the RCAR proteins *in vitro* (Antoni et al., 2012; Dupeux et al., 2011). However, in the present study AHG1 interacted with RCAR1, RCAR3, RCAR5 and RCAR9 in the presence of ABA, indicating an RCAR-dependent regulation of AHG1 activity. In future, this physical linkage could be useful in biotechnological control of seed germination traits (Ben-Ari).

HAI1 was previously reported to be a golgi-localized PP2C associated to leaf senescence (Zhang et al., 2012b). Such a specific localization could interfere with Y2H testing and might explain the negative results. However, the studies of Bhaskara et al. (2012) and Zhang et al. (2012a) disputed such a localization and showed a nuclear localization for HAI1. Additional interaction studies with the three HAI PP2Cs revealed weak constitutive interaction of HAI1 with RCAR3 and RCAR4 and also ABA-induced interaction with RCAR8 (Bhaskara et al., 2012). Thus, the disputed localization of HAI1 in the report of Zhang et al. (2012b) might be due to degradation of mistargeted protein. The use of a c-terminally fused fluorophore in the study might have interfered with the c-terminal NLS (Antoni et al., 2012) and caused improper targeting. Nevertheless, these contradictory results could also explain, at least in part, the absence of HAI1 interaction in yeast. Even though the interaction of HAI1 with RCARs is absent or very weak in yeast (Bhaskara et al., 2012), an inhibitory effect of some RCAR proteins on HAI1 has been shown *in vitro* (Antoni et al., 2012).

The different interaction patterns might at least be in part based on the sequence-specific differences of the nine clade A PP2Cs. In Fig. 47 the structural similarity and characteristic differences at the amino acid level within distinct domains are depicted.

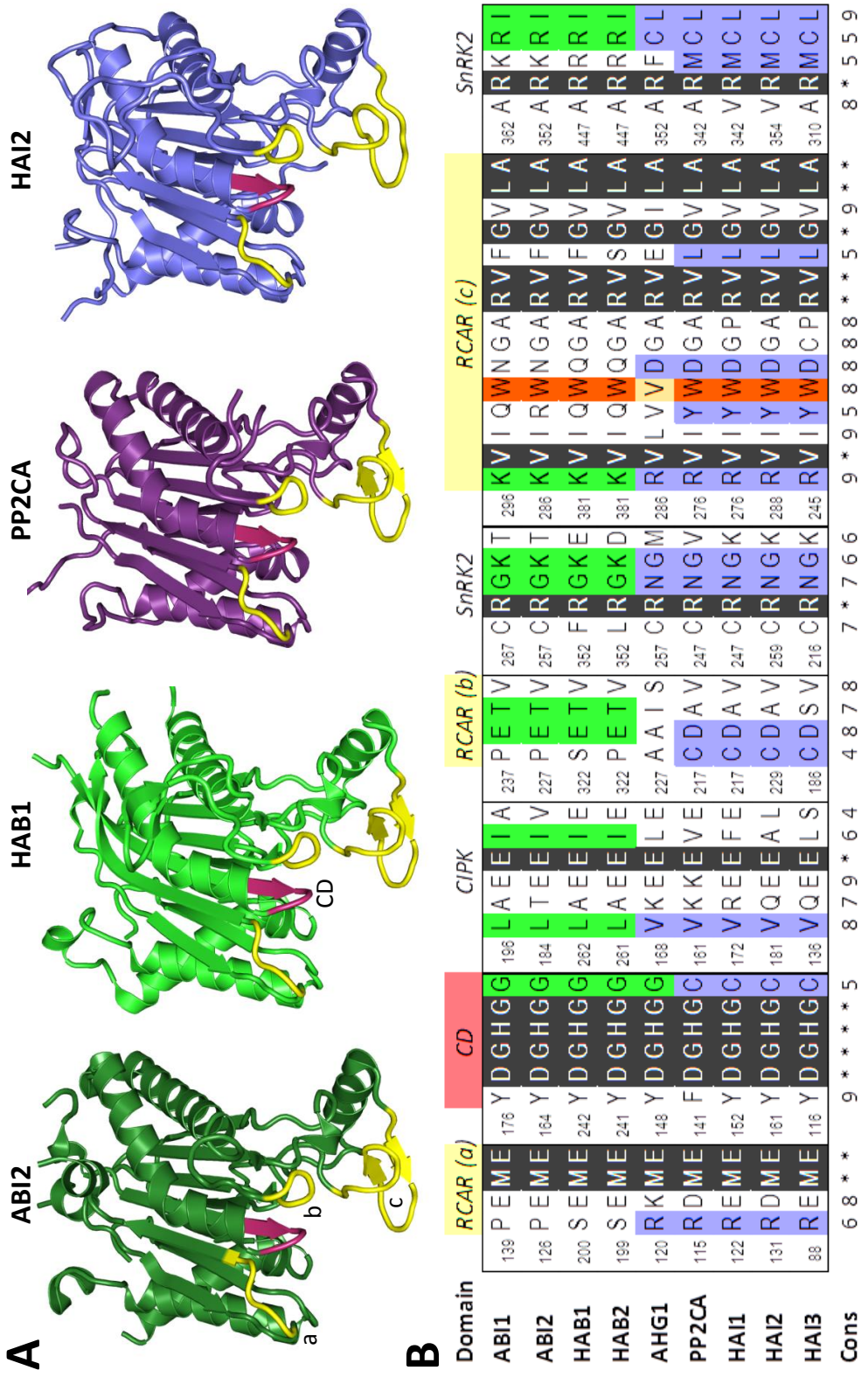


Fig. 47: Structures and alignment of PP2C proteins. (A) The structures of ABI2, HAB1, PP2CA and HAI2 are shown based on the PDB datasets 3UJK and 3UJG (green) and HHpred structural predictions (purple), respectively. The catalytic domain (CD) and loops involved in RCAR interaction are highlighted in pink and yellow, respectively. (B) Important PP2C residues involved in RCAR (RCAR *α-c*), SnRK2 and CIPK interaction as well as the catalytic domain (CD) are aligned. The numbers indicate the amino acid position. The bottom line evaluates conservation (Cons) of critical residues within the family from 0-not conserved to *-completely conserved (dark shading). The coloring corresponds to characteristic residues conserved in ABI1-like (green) or HAI group PP2Cs (purple). The conserved Trp (W) involved in ABA coordination is highlighted in orange.

The structures of vital phosphatase domains are conserved among the clade A PP2Cs (Fig. 47A). Especially the loops and helices involved in RCAR docking are strikingly similar in their spatial orientation. On the amino acid sequence level the phosphatases reveal a more distinct pattern of conservation (Fig. 47B). Apart from the highly conserved residues in each of these domains, there are also clear differences, especially between the closely related ABI1-like PP2Cs and the homologous HAI PP2Cs. For instance, the a-loop, which is important for RCAR coordination, contains a conserved, basic Arg (R) residue only in the HAI group phosphatases. On the other hand, the ABI1-like PP2Cs possess a basic Lys (K) residue in the SnRK2 interaction domain. In the HAI PP2Cs, this residue is replaced by a hydrophilic Gly (G). Hence, not only the RCAR interaction domains, but also the residues involved in SnRK2 and CIPK interaction feature characteristic differences. AHG1 is unique among the clade A PP2Cs in its amino acid sequence at some critical positions. For example, the important tryptophane (W) directly involved in ABA coordination in the other PP2Cs (Melcher et al., 2009) is replaced by a valine (V) in AHG1 (see Fig. 47B). However, the interactions observed for AHG1 in the present Y2H studies indicate, that the presence of the Trp-lock is not required for the formation of complexes with certain RCARs (Weiner et al., 2010).

Generally, the alterations in interaction-mediating residues between ABI1-like and HAI1-like PP2Cs could be a limiting factor for the control of PP2Cs by the RCARs and the regulation of downstream targets by the PP2Cs. To estimate the biochemical variations governing preferential complex formation, the varying interfaces of PP2Cs and RCAR proteins are illustrated in Fig. 48. Based on the alignments provided in Fig. 46 and Fig. 47, critical residues, which are divergent between PP2C and RCAR subfamilies, are highlighted. For instance, the ABI2 interaction interface contains two prominent, hydrophobic Pro (P) residues. In the HAI subfamily, these residues are replaced by hydrophilic Cys (C) and Arg (R) residues. Steric hindrance and ionic interaction might be responsible for the establishment of certain co-receptor complexes. Minor changes in the interface result in the complex molecular regulation of the PP2C interaction. Similarly, the docking and ligand coordination sites in the RCAR proteins possess different biochemical properties (Fig. 48, C and D). Ionic and hydrophobic interactions are vitally important for the establishment of physical contact between PP2Cs and RCARs after ligand perception (Peterson et al., 2010; Shibata et al., 2010; Yin et al., 2009). Despite their overall similarity, the RCAR proteins from subfamilies I and III possess characteristic differences in important domains. These specific deviations influence dimerization, ligand binding and the eventual inhibition of the PP2Cs.

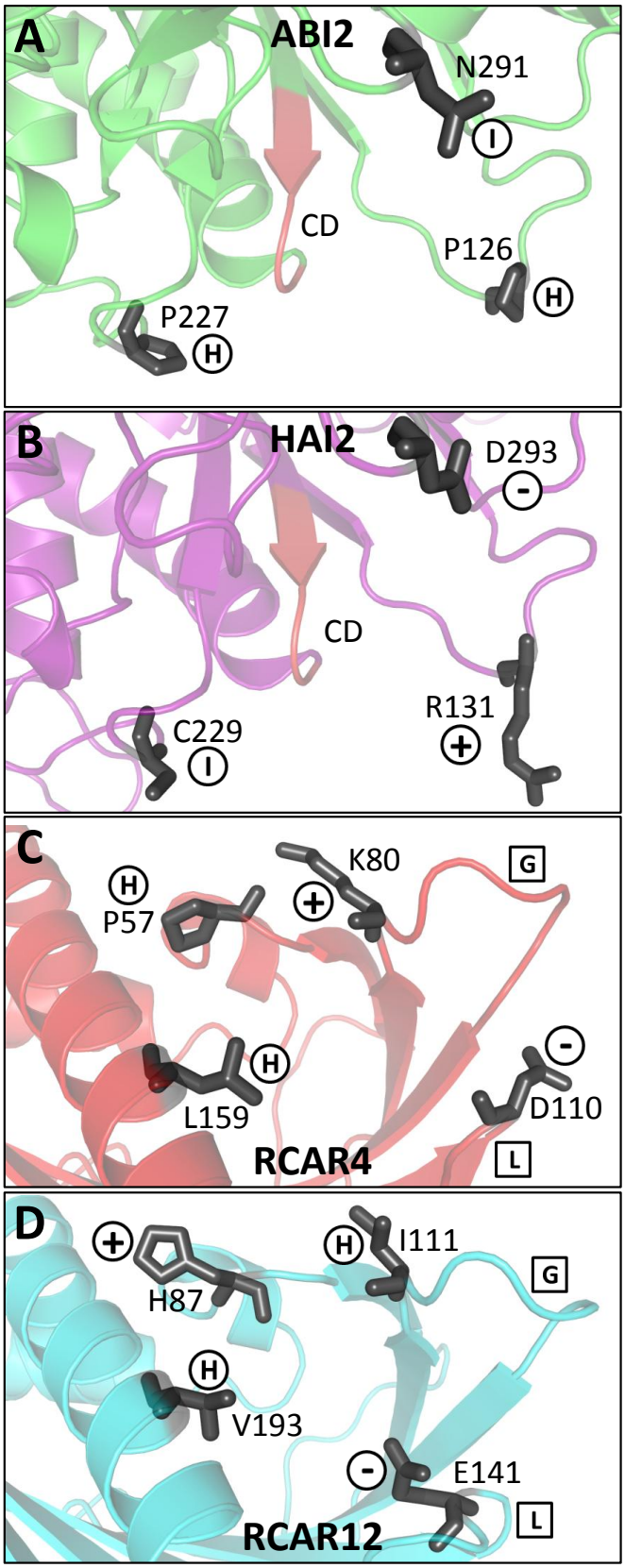


Fig. 48: Close-up views of the PP2C catalytic site and the RCAR interaction surface. The interfacing sites of ABI2 (A) and HAI2 (B) PP2Cs are depicted with important, divergent residues highlighted in grey. The ligand binding cavities of RCAR4 (C) and RCAR12 (D) sensory proteins are depicted with important, divergent residues highlighted in grey. Hydrophobic (H), hydrophilic (I), positively charged (+) and negatively charged (-) amino acid residues are marked. The catalytic domain (CD) of the PP2Cs is highlighted in red. The "gate" and "latch" loops of the RCAR proteins are labeled [G] and [L].

To estimate the significance of the results gathered in the heterologous studies *in vivo*, the PP2C and RCAR components were tested in Arabidopsis protoplasts. Protoplasts are a fast and versatile system to study signal transduction pathways by transient gene expression (Yoo et al., 2007). Ectopic expression of genes involved in ABA-dependent signaling was used to functionally characterize ABA signaling regulators earlier (Himmelbach et al., 2002; Ma et al., 2009; Moes et al., 2008; Sheen, 1998). Finally, this system allowed the reconstitution of a signaling cascade from the receptor to the transcriptional regulator using a minimal set of components (Fujii et al., 2009). Based on the Y2H vectors used in the interaction analyses, a compatible acceptor vector for protoplast expression was generated. Transient ectopic expression was achieved by placing the PP2C and RCAR sequences under the control of the strong viral 35S promoter. This simple "cut and paste" strategy allowed fast clonding and testing of all nine PP2Cs and all 14 RCARs for their ABA-related signaling function *in vivo*. For the first time, there is clear evidence for all 14 RCARs to positively regulate ABA-dependent gene expression. RCAR over-expression markedly increased ABA response, visualized as induction of an ABA-responsive reporter. Contrastingly, ectopic expression of all clade A PP2Cs diminished ABA-related signal transduction, confirming their role as major negative regulators.

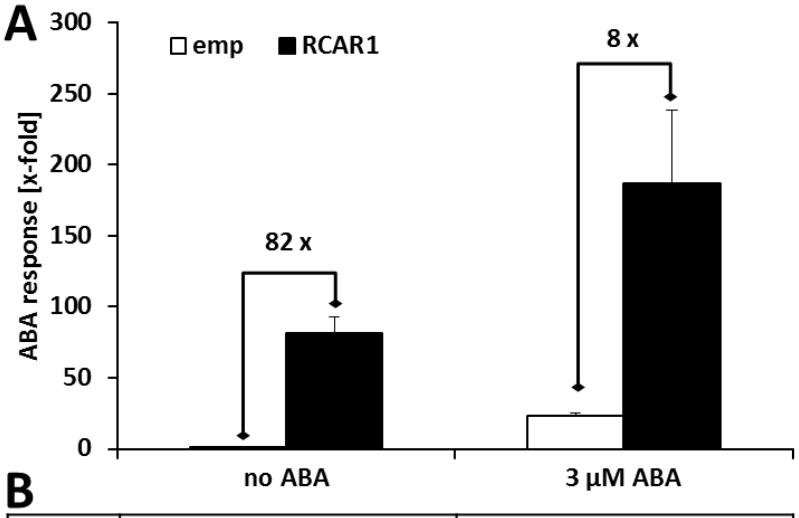
3.1.3 Relevance of PP2C-RCAR coupling *in vivo*

In the protoplast assays, clear differences in the extent of signaling activation or repression were observed upon co-expression of different RCARs or PP2Cs, respectively. Strikingly, the interaction preferences of the RCARs for certain PP2Cs, which were observed in yeast, are consistent with the functional variation found in protoplasts.

RCAR1 expression alone, for instance, markedly increased ABA response to ~80-fold. This induction could be boosted by addition of exogenous ABA up to about ~180-fold, relative to the untreated control. The co-expression of PP2Cs suppressed this RCAR1-mediated increase, particularly in the absence of exogenous ligand. In the presence of ABA, PP2CA and HAI3 were able to repress signaling down to control levels. HAI2 did not completely abolish RCAR1-elicited increases. This could be explained by the strong interaction of RCAR1 with HAI2 observed in yeast, leading to an efficient inactivation of PP2C acitivity. For RCAR8 the situation was different. RCAR8 alone caused a signaling response similar to RCAR1 without exogenous ABA. In the presence of the ligand, approximately the same levels of induction were seen. So, excessive concentrations of the ligand did not increase the signal, which indicate a situation with endogenous ABA levels close to saturation of the system. Also the effect of PP2C co-expression together with RCAR8 was different from RCAR1. HAI2 was able to completely block RCAR8-mediated signaling increases, but ABI1 only marginally counteracted RCAR8-dependent induction. This could be the result of a strong interaction of RCAR8 with ABI1 and a

weaker interaction with HAI2, which is consistent with the results in the Y2H analyses. RCAR8 might completely inactivate ABI1 protein, whereas a part of the ectopic HAI2 phosphatase was still free to block downstream signaling due to incomplete RCAR8 interference. Strikingly, over-expression of the subfamily III member RCAR11 alone was not sufficient to significantly induce ABA response signaling. The supplementation of exogenous ABA induced the signal up to values similar to RCAR8 (74-fold and 66-fold, respectively). Without exogenous ligand RCAR11 only caused minimal reporter induction (~5-fold). Co-expression of ABI2 together with RCAR11 did not significantly reduce ABA response. HAI1 PP2C reduced RCAR11-mediated signaling to approximately 50 % (from 66-fold to 30-fold). ABI2 might be efficiently deactivated by RCAR11. As HAI1 did not interact with RCAR11 in the Y2H assays, the phosphatase could block ABA downstream signaling, undisturbed from ectopic RCAR11 protein.

In conclusion, the different affinities for ligand binding and PP2C interaction may result in a discriminable effectiveness of the single RCAR proteins to elicit ABA-related signaling. Of course, this effect depends also on the presence of the respective PP2C counterparts in the cell. In order to estimate the extent of this implication, the RCAR-dependent ratios of ABA response induction compared to the samples without RCARs were calculated.



B

RCAR1	81,8	8,1
RCAR8	47,9	4,6
RCAR11	4,6	3,3

Fig. 49: RCAR-dependent induction of ABA response in protoplasts. (A) The ratios of signaling induction relative to empty vector (emp) controls in the absence (no ABA) or presence of 3 µM ABA are shown for RCAR1. (B) The ratios of induction relative to empty vector (emp) controls in the absence (no ABA) or presence of 3 µM ABA are calculated for RCAR1, RCAR8 and RCAR11 based on the experiments from Fig. 15.

Fig. 49 shows the levels of induction of the ABA-responsive reporter caused by an expression of RCAR1, RCAR8 or RCAR11. The ratios are relative to the corresponding controls without RCAR co-expression. RCAR1, representing subfamily I, caused a tremendous 80-fold increase of the ABA response in the absence of the ligand in relation to untreated controls without any RCAR1. This RCAR1-dependent induction was reduced to only 8-fold in the presence of ABA. RCAR8 only caused a 48-fold or 5-fold increase in the absence or presence of ABA, respectively. In contrast to this, subfamily III member RCAR11 did not exhibit such a strong induction in the absence of the ligand. Both with and without ABA the signal was about 4-fold increased after RCAR11 co-expression. These distinct ratios of signaling modulation by the RCARs might correspond to the different affinities for ABA binding. Upon ABA perception the RCARs could trigger PP2C inactivation and the release of PP2C downstream factors to mediate ABA-dependent responses. RCARs from subfamily I and II show high affinities for the ligand in the low nanomolar range (Ma et al., 2009; Szostkiewicz et al., 2010), while RCARs from subgroup III show lower binding affinities (Melcher et al., 2010b; Soon et al., 2012b). In combination with the ligand-independent regulation of PP2Cs by monomeric RCARs (Hao et al., 2011), the present results confirm that the RCARs from subgroups I and II are high-affinity ABA receptors. The RCARs from subfamily III seem to require higher ABA concentrations to elicit the PP2C inactivation-dependent increase in ABA response. This might be due to the lower affinity and the dimeric state reported for this group (Dupeux et al., 2011; Nishimura et al., 2009).

These observations in protoplasts were further confirmed by the comparison of HAI3-dependent effects on different RCAR subfamily members. RCAR1 from subfamily I, as well as RCAR5 and RCAR8 from subfamily II, induced ABA response signaling in protoplasts in the absence of exogenous ligand. RCAR1-mediated induction was further increased by exogenously supplying the ligand. For RCAR5 and RCAR8 providing the ligand did not additionally alleviate the signal, indicating a saturation of the system. The receptors of subfamily III did not cause an induction of ABA-dependent signaling without exogenous ABA, but only in the presence of exogenous ABA. The co-expression of the phosphatase HAI3 globally repressed the ABA response, especially for RCAR12, whose inductive effect was widely terminated. RCAR5- and RCAR8-mediated increases were markedly reduced compared to the samples without PP2C. Hence, the effects observed in protoplasts again corroborate the Y2H results indicating a strong interference of RCAR1, RCAR5 and RCAR8 with HAI3 also *in vivo*.

The inductive effect of different RCARs was further studied by varying the amount of expression vectors transfected in protoplast experiments. Again, a clear difference in function could be observed for RCAR3 from subgroup I and RCAR12 from subgroup III. For instance, RCAR3 expression alone already stimulated a strong increase in ABA response, irrespective of the ligand ABA. Only at low RCAR3 concentrations the supplementation of the ligand doubled the RCAR-elicited signal induction.

This might indicate that the system is saturated by the expression of the receptor alone and the residual, endogenous ABA. For RCAR12 the situation is different. In the absence of the hormone, no induction of the ABA-responsive reporter was observed. With the addition of the ligand, an RCAR12-dependent increase of ABA-related signaling was visible. To study the inhibitory effects of the PP2Cs on ABA signaling, similar protoplast titration experiments were performed. RCAR5-mediated signal induction was inhibited by different PP2Cs. The results corroborated the idea of preferential interaction also *in vivo*. Based on their characteristic coupling in yeast, RCAR5 interferes differentially with these three phosphatases. AHG1 efficiently blocks RCAR5-induced reporter activation at low protein levels. HAI1, which is not interacting with RCAR5 in yeast, is similarly effective. PP2CA showed different behavior, as only high levels of the phosphatase inhibited RCAR-mediated ABA response. These results are consistent with the findings that the RCAR proteins have different binding affinities for ABA and the PP2Cs (Antoni et al., 2012; Szostkiewicz et al., 2010). Furthermore, they emphasize the importance of the combinatorial assembly of variable co-receptor complexes to fine-tune ABA response.

3.2 Transcriptional regulation of signaling components

The phytohormone ABA causes vast transcriptional alterations in Arabidopsis (Busk and Pages, 1998; Gosti et al., 1995; Skriver and Mundy, 1990; Wang et al., 2011). The major components in the ABA signaling cascade are tightly regulated at the transcriptional level. Among those important factors are the PP2Cs from clade A. As central negative regulators of ABA-mediated signaling pathways, the investigation of their transcriptional regulation is vital to understand their function *in planta*. Analyses of the publicly available microarray datasets illustrated that clade A PP2Cs show a characteristic pattern of regulation throughout development and in the course of stress perception and response.

The heat-maps depicted in Fig. 18 and Fig. 19 visualize the transcriptomic modulations of clade A PP2C genes throughout development. The regulation is shown as relative induction or repression compared to a developmental baseline value of all samples. To estimate the abundance of the PP2Cs compared to a constitutively expressed reference gene, the baseline values were calculated relative to At4g05320 UBQ10 (values in the control columns of developmental samples). These ratios already indicate a minimal expression of all clade A PP2Cs throughout development. Only at the very late stages, e.g. in a senescent leaf, there is a strong up-regulation of most of the PP2Cs (see Fig. 19). Zhang et al. (2012b) reported that HAI1 controls water loss during senescence. This proposed function of a PP2C in old, drying plant tissues is corroborated by the HAI1 expression pattern in seed maturation and germination. At the late stages, i.e. mature and dry seeds, also other PP2Cs are

found to be expressed even at higher levels than the constitutive UBQ10 control gene. Especially AHG1 and the HAI PP2Cs are markedly induced in the course of seed maturation (up to ~350-fold). Thus, besides the important functions of reported for AHG1 and PP2CA in germination control (Nishimura et al., 2007; Yoshida et al., 2006b), also other PP2Cs seem to be important for proper seed maturation and subsequent germination. As the process of germination is cardinally controlled by ABA, PP2C transcription is directly inversely correlated to the progression of germination.

The direct regulation of PP2C gene transcription is by different stress regimes is depicted in Fig. 20. PP2C expression increases rapidly (< 1 h) after exposure to osmotic, salt or drought stress. The transient and weak response (< 10-fold induction) after exposure to drought stress is due to the experimental setup. The seedlings were only transiently exposed to drought for 15 min. Therefore, the stress-responsive system was only partially activated, before being reset to normal conditions. As all the stress situations applied are, at least partly, mediated by ABA, the effect of the phytohormone itself on PP2C transcprits was also investigated. The transcription levels of the PP2Cs responded expeditiously and strongly to exogenous ABA (compare Fig. 21). The application of other hormones such as ethylene or auxin did not significantly influence the PP2C transcript abundance. Therefore, the transcriptional regulation of PP2C transcripts throughout development and in stress response signaling cascades seems to be mainly and specifically mediated by ABA. These findings reflect the strong induction of clade A phosphatases by ABA and ABA-mediated stress signaling pathways. For instance, the HAI PP2Cs were reported to be 59- to 270-fold upregulated after a 6 h treatment with 50 µM ABA (Fujita et al., 2009). Previous studies on the ABA-mediated up-regulation of PP2C transcription suggested that this mechanism serves as a negative feedback to desensitize the plant after stress relief (Merlot et al., 2001; Valdes et al., 2012).

The expression of the regulatory subunit of the ABA-receptor complex, i.e. the RCAR, is inversely correlated to ABA-signaling (Fig. 22). The transcripts of the RCAR genes are significantly reduced in the course of stress treatments and also after supplementation of the media with exogenous ABA. This ABA-dependent down-regulation could be part of the desensitizing function suggested for the induction of the clade A PP2Cs on the transcriptional level (Hoth et al., 2002; Merlot et al., 2001; Saez et al., 2006). Similar to PP2C transcription, RCAR transcription is not significantly influenced by other phytohormones, indicating a discrete signaling pathway. Nevertheless, a direct connection of RCAR10 protein in the regulation of JA signaling was proposed (Lackman et al., 2011).

3.3 Regulation of downstream targets by clade A PP2C master regulators

3.3.1 Control of CIPK function by PP2Cs

CIPKs are indirectly regulated and activated by Ca^{2+}. Their prominent role is the activation and regulation of channels and transporters in multiple signaling pathways (Batistic and Kudla, 2004; Pandey, 2008). A direct interconnection of clade A PP2Cs and CIPKs was first shown by Ohta et al. (2003) and Guo et al. (2001). In an attempt to pursue these initial findings, a compilation of possible PP2C-CIPK interactions was systematically analyzed in yeast. The present study revealed a specific pattern of interaction, and hence a direct physical link between clade A PP2Cs and CIPKs.

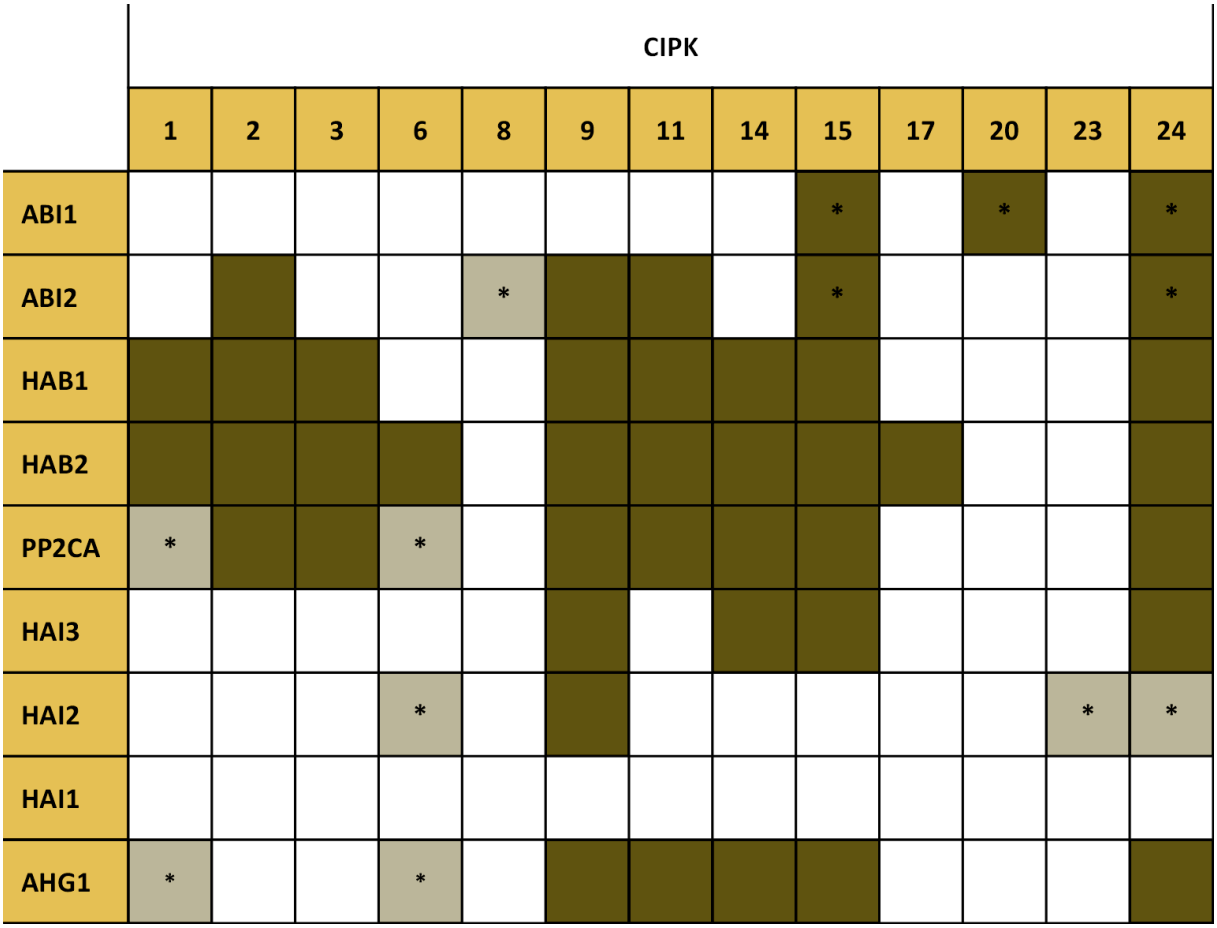


Fig. 50: Summary of PP2C-CIPK interaction. The interactions between clade A PP2Cs and CIPKs observed in yeast are indicated by dark shading. Interactions reported earlier are marked with an asterisk. Light shading indicates interactions found in BioGrid database but not in the present analyses (Chatr-Aryamontri et al., 2013).

The binary interactions observed in the Y2H growth assays are summarized in. The results are to a large extent consistent with the interactions previously published by Guo et al. (2002), Lee et al. (2007) and Ohta et al. (2003) (compare Fig. 25). However, the interaction of HAI2 with CIPK23 shown by Lee et al. (2007) could not be reproduced in the system used. Additional PP2C-CIPK complexes were identified in a screen for regulatory components of the potassium transporter AKT1 (Lan et al.,

2011). This group investigated the interaction of HAI2 with CIPK6, CIPK16, CIPK23 and CIPK24 in the control of AKT1 activity as downstream target (Xu et al., 2006). Furthermore, AHG1 and PP2CA interacted with CIPK1 and CIPK6. Moreover, AHG1 also interacted with CIPK16 in these assays. These findings illustrate the model of a functional interplay of a protein kinase (CIPK) - phosphatase (PP2C) pair governing the activitiy of downstream effectors (Lan et al., 2011; Sanchez-Barrena et al., 2013).

The assays performed in the course of this work showed previously unknown interaction of CIPK1 with HAB1 and HAB2 but not with PP2CA or AHG1. Similarly, an interaction was detected for CIPK6 with HAB2 but not for CIPK6 with HAI2, PP2CA or AHG1. In general, the results of Y2H analyses may vary in their read-out depending on the yeast strain, the reporter system and the plasmids used. However, there is a clear, direct connection of PP2Cs from clade A with CIPKs. Considering the reports from Lan et al. (2011), Lee et al. (2007), Ren et al. (2013) and Du et al. (2011), a multiprotein network might be regulating the downstream targets, e.g. AKT1. Namely, the PP2Cs were shown to interact with CIPKs, CBL Ca^{2+}-sensors and the channel itself (Lee et al., 2007). On the other hand, CIPKs phosphorylate both the CBLs and the channel to regulate their function (Du et al., 2011; Xu et al., 2006). The control of the phosphorlyation status of these downstream components by the PP2Cs seems to be the major switch in the regulation of the whole signaling cascade. Therefore, the regulatory cascade involved in the regulation of CIPK23 and subsequent AKT1 activation might also be similar for other CIPKs and their downstream targets.

Based on the heterologous interaction studies, the relevance of this interconnection was examined *in vivo*. The effect of ectopic CIPK protein levels on ABA-dependent signal transduction was investigated by transient expression in protoplasts. Experiments with wt CIPK constructs did not produce any significant effects (data not shown). This might be due to the inactive state of CIPKs under normal conditions. Only upon Ca^{2+}-mediated activation via CBL-proteins the CIPKs get activated. To achieve CBL-independent activation of CIPKs, a conserved threonine (T) residue in the activation loop of the protein kinases was replaced with an aspartate (D). This point mutation generates constitutively active versions of the CIPKs for *in vitro* and *in vivo* studies (Gong et al., 2002; Guo et al., 2004). Gao et al. (2012) could show an about 10-fold higher activity for CIPK3 T183D mutant protein compared to wt CIPK3 in their *in vitro* studies. Ectopic expression of CIPK2 produced minor increases. CIPK11 and CIPK14 caused a minor decrease in the ABA response. The other CIPKs did not cause any significant alterations. The effects of CIPK2, CIPK11 and CIPK14 could be augmented by the use of protoplasts derived from the ABA-deficient mutant *aba2*, which is usually hypersensitive to ABA-related stimuli (Lin et al., 2007; Schwartz et al., 1997). Considering the known functions of CIPK family members, a direct control of ABA-mediated transcriptional control seems unlikely. The CIPK-mediated effect on

ABA-related gene regulation might be secondary, possibly due to a feed-back control. However, CIPK14 was to a large extent able to counteract RCAR12-mediated increases in ABA-dependent gene regulation (see Fig. 29). While exogenous ABA did not significantly influence the CIPK-mediated effects, over-expression of CIPK14 clearly hampered RCAR-dependent signaling activation. The inhibition of certain clade A PP2Cs by RCAR12 might release the CIPKs from PP2C control. This could indicate, that the PP2Cs from clade A block CIPK function on downstream targets. Accordingly, the effects caused by an over-expression of CIPK2, CIPK11 and CIPK14 were completely abolished by co-expression of PP2CA. PP2CA interacted directly with all three protein kinases in yeast (Fig. 25). So, a co-expression of the PP2C might counteract the CIPK-related phosphorylation events and block downstream effects.

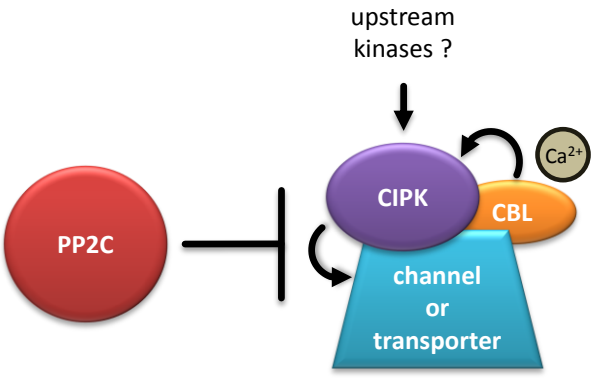


Fig. 51: Model for the regulation of CIPK signaling. The Ca^{2+}-dependent binding of CBL protein causes activation of CIPK protein kinases and subsequent phosphorylation of downstream targets such as channels or transporter. CBLs, CIPKs and downstream targets interact with each other and the PP2Cs. The involvement of unknown upstream kinases remains elusive.

The function of CIPKs in cellular signaling is known for only a few members. The PP2C-CIPK interactions discovered in this work might help to understand the physiological function of the respective protein kinases. For instance CIPK1 was reported to be involved in ABA-dependent signaling processes (D'Angelo et al., 2006). The interactions documented here and by Lan et al. (2011) could provide a direct interconnection. The same holds true for CIPK3. ABA- and salt-hypersensitive phenotypes were reported for CIPK3 knock-out plants in Arabidopsis (Pandey et al., 2008) and homologous mutants in rice (Piao et al., 2010). The physiological role of CIPK9 is attributed to potassium uptake (Pandey et al., 2007). Similar to CIPK3, CIPK9 transcription is induced upon ABA-related stress treatments (Kim et al., 2003; Pandey et al., 2007; Pandey et al., 2008). ABA-hypersensitive phenotypes were also reported for CIPK15 mutant plants (Guo et al., 2002) and could indicate a negative regulation of ABA responses by certain CIPKs. This would further be consistent

with the results observed in the transient protoplast expression assays, where CIPK11 and CIPK14 lead to a repression of ABA-dependent signal transduction (see Fig. 28). Studies on CIPK14 showed that it interacts with the sensory proteins CBL2, CBL3 and CBL8, which are mediating membrane localization (Batistic et al., 2010). Lee et al. (2005) proposed an involvement of CIPK14 in sugar sensing and signaling. Interestingly, the work of Akaboshi et al. (2008) on the crystal structure of a CBL2 sensory protein revealed that the co-cristallized regulatory domain of CIPK14 showed homology to At1g24000. This gene is a member of the MLP superfamily and structurally closely related to the RCAR proteins. Such a structural similarity is reminiscent of the molecular mimicry existing for RCARs and SnRK2s in PP2C interference (Soon et al., 2012a).

In conclusion, the interactions between PP2Cs and CIPKs identified in this work provide a direct connection of ABA-related signaling components and downstream effector proteins. The phenotypes observed suggest that CIPKs might be part of the machinery controlling ion homeostasis. Direct physical interaction could interconnect PP2C function and the regulation of ion fluxes via the CIPKs.

3.3.2 Functional relation of PP2Cs and CPKs in ABA signaling

Previous reports on CPKs involved in ABA-related processes provided evidence for a close link of these Ca^{2+}-regulated protein kinases to core ABA signaling (Sheen, 1998; Zhu et al., 2007). For example, CPK10 was shown to be involved in the stomatal control in response to drought stress (Zou et al., 2010). The studies of Geiger et al. (2009) and Geiger et al. (2011) revealed that particularly CPK21 controls ion fluxes via the regulation of channel activities. The reconstitution of a signaling module with CPK21, the SLAC1 ion channel, the PP2C ABI1 and the PP2C regulating RCAR1 in a heterologous system confirmed these findings (Geiger et al., 2010). Lynch et al. (2012) showed that CPKs directly interacted with clade A PP2Cs in the regulation of Arabidopsis ABA signaling. Namely, CPK11 interfered physically with PP2Cs in the control of ABF-dependent transcriptional regulation.

Besides CPK21, also closely related CPK23 is known to be involved in a number of ABA-related traits (Geiger et al., 2010; Ma and Wu, 2007). To investigate a possible connection of CPK23 with clade A PP2Cs, extensive interaction analyses were performed in a Y2/3H heterologous system. Indeed, a direct physical interaction was identified between a clade A PP2C, i.e. PP2CA, and a constitutively active version of CPK23. Under normal conditions an auto-inhibitory domain in the CPK protein blocks the catalytic center (Harper et al., 1994; Wernimont et al., 2010). A truncation of this auto-inhibitory domain mimics the Ca^{2+}-induced conformational changes necessary for CPK activation. The resulting protein is deregulated and constitutively active. In the present studies, an activated version of CPK23 interacted with PP2CA, and to a lesser extent also with other clade A PP2Cs (see Fig. 31). As

a control an inactive, kinase dead version of the truncated CPK, harboring a deleterious mutation in its kinase domain, was included in the analyses. It showed no interaction. Hence, the interaction of the PP2C and CPK23 clearly depends on its protein kinase activity.

For a detailed understanding of this interaction in the context of the core ABA signaling module, the PP2C-CPK interaction was also challenged by co-expression of a competitive third protein partner, i.e. an RCAR. These results indicate that RCAR proteins are strongly competing for PP2C interaction with the protein kinase. The regulatory components could replace the CPK from its interaction with the PP2C even at low levels, implicating a higher binding affinity of the RCARs. Conversely, the active CPK23 (CPK23t) was only at very high levels able to limit PP2C-RCAR9 interaction. Additionally, this effect was again dependent on the kinase activity of the truncated CPK23. A kinase dead version (CPK23td) did not show such an inhibitory effect. In conclusion, only the active kinase is able to interfere with PP2C-RCAR interaction.

Similar results were obtained by the competition of CPK23t with SnRK2 protein kinases for the PP2C interaction. SnRK2s are major downstream targets of the PP2Cs (Nakashima et al., 2009). High levels of the SnRK2s interrupted the PP2C-CPK interaction (see Fig. 35). Lower levels of SnRK2s did not significantly prevent PP2CA-CPK23 coupling. Interestingly, the interfering effect of SnRK2.2 was stronger compared to SnRK2.6, which is consistent with the strengths of PP2C-SnRK2 interactions reported by Umezawa et al. (2009). Furthermore, the kinase dead version of SnRK2.6 (SnRK2.6d) behaved like the wild type. This indicates that the protein kinase function of this SnRK2 is not necessary for the interference with CPK23 interaction. The influence of CPK23t co-expression on PP2C-SnRK2 interaction resembles the situation of RCAR-CPK competition. High levels of the active version of CPK23 prevented the coupling of ABI2 with SnRK2s (Fig. 35). The Y3H analyses indicate overlapping interaction interfaces for CPKs and PP2C interactors, such as RCARs and SnRK2s. Furthermore, only the active kinase was able to interfere with RCAR and SnRK2 interaction.

In conclusion, there is functional competition between CPK23 and PP2C (pseudo)substrates. As this interference is clearly dependent on the protein kinase activity, the CPK may be a crucial regulator of the core ABA signaling relay. The CPKs could directly modulate PP2C-dependent signal transduction. Furthermore, the interactions detected for the active version of CPK23 and the clade A PP2Cs confirm the results of Geiger et al. (2010) and Scherzer et al. (2012) for CPK21 and CPK23. A plausible explanation could also be a kinase-dependent effect on the interaction features of the PP2C or the partner protein. Still, a reason for the kinase activity-dependent effect might be the improper folding of the kinase dead version resulting in impaired binding. Such a kinase-related modification of PP2C

function was already shown for PPM1E, a human PP2C isoform, which is phosphorylated and thereby activated by a Ca^{2+}/calmodulin-dependent protein kinase CaMKI (Onouchi et al., 2012). The increased phosphatase activity of the phosphorylated PPM1E leads to a stronger inhibition of downstream targets. The results of Lynch et al. (2012) suggest a similar mode of regulation for the PP2Cs from clade A by CPK11 in Arabidopsis. Their studies provide evidence for a direct regulation of both, the PP2Cs and ABF transcription factors, by CPK11 in the regulation of ABA-related signal transduction. Summing up, CPKs might be a point of integration for Ca^{2+}-dependent signaling and ABA-mediated abiotic stress signaling. The model in Fig. 52 illustrates the possible connection of ABA signaling and CPK function. Under unstressed conditions the CPKs remain inactive due to autoinhibition and a PP2C-dependent restraint. After stress perception, CPKs could balance Ca^{2+}-dependent and PP2C-related ABA signaling (Zhao et al., 2011).

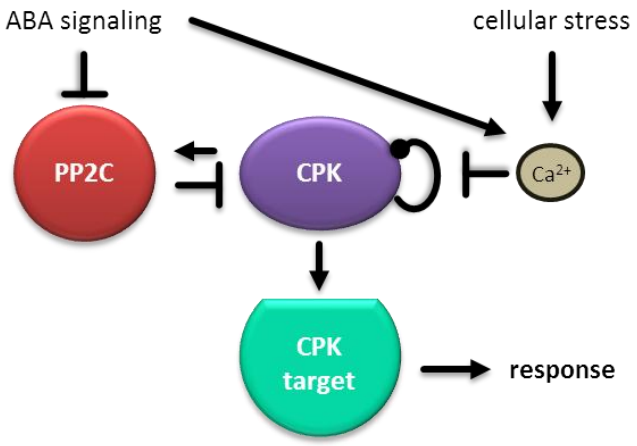


Fig. 52: Model of CPK signaling. The Ca^{2+}-dependent release of CPK activity is counteracted by clade A PP2Cs. In the presence of ABA, PP2Cs are withdrawn from the system by RCAR-mediated inhibition. In turn, CPKs are free to phosphorylate downstream targets and to modulate PP2C function.

These observations on CPK-related signal transduction are in line with the PP2C-dependent control of other downstream kinases, e.g. CIPKs and SnRK2s (Brandt et al., 2012; Lee et al., 2007; Lee et al., 2012; Vlad et al., 2010; Xie et al., 2012). The studies of Lynch et al. (2012) provided first evidence for a CPK-dependent signal transduction cascade resulting in the phosphorylation and activation of ABF TFs. Such a phosphorylation-dependent switch might be counteracted by clade A PP2Cs (Yoshida et al., 2010). The preliminary results on the PP2C-ABF interaction here and the results by Lynch et al. (2012) closely connect the transcriptional regulation to the mutual regulation exerted by a PP2C-CPK switch. Therefore, CPKs could play an important role in the integration of Ca^{2+}-related signaling and ABA-dependent stress signaling.

3.4 Regulation of non-clade A PP2Cs by RCAR-related MLPs in stress signaling

The proteins most closely related to RCARs are the MLPs (see Fig. 37 for phylogeny). Both RCARs and MLPs share the typical Bet v 1 helix-grip fold and possess an internal cavity suitable for the binding of diverse ligands (Lytle et al., 2009). Apart from their structural similarity to the RCARs, the physiological function of the MLPs remains largely unknown. Diverse reports on MLPs from different species indicated their involvement in fruit abscission, bolting and salt stress response (Chen and Dai, 2010; Guo et al., 2011; Ruperti et al., 2002). Some of the MLPs were shown to be capable of binding small hydrophobic substances (Lytle et al., 2009; Radauer et al., 2008). Exemplary structures of several Bet v 1 related proteins are shown in Fig. 53.

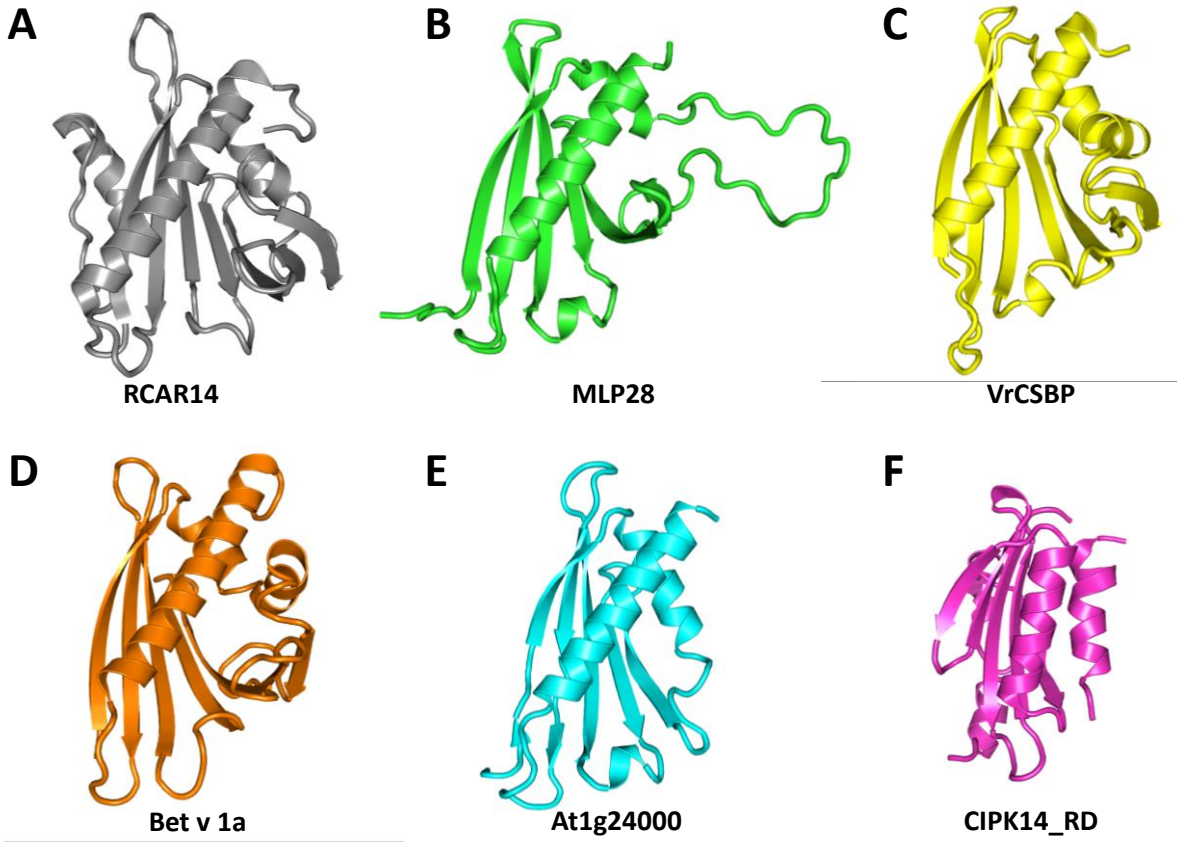


Fig. 53: Examples of RCAR-related proteins. The structures of RCAR14 (PDB code: 3KDH), At1g70830 MLP28 (2I9Y), *Vigna radiata* Cytokinin Specific Binding Protein (VrCSBP, 2FLH), *Betula pendula* major birch pollen allergen α (Bet v 1a, 4A85), At1g24000 MLP-related protein and the regulatory domain of CIPK14 (CIPK14_RD, 27FD) are depicted. For further details see text.

The members of the MLP superfamily share a similar structural fold with a central cavity for binding hydrophobic substances (Lytle et al., 2009). For instance, RCAR14 was shown to bind ABA (Yin et al., 2009). Under the crystallization conditions used by Lytle et al. (2009), MLP28 engulfed the human steroid hormone progesterone. Cytokinin-specific binding led to the identification of a *Vigna radiata*

protein, termed CSBP (Pasternak et al., 2006). Bet v 1 and its relatives were shown to be capable of integrating kinetin or deoxychelate in their central cavity (Kofler et al., 2012). The structures of At1g24000 (Levin et al., 2007), an MLP-related protein, and the regulatory domain of CIPK14 (CIPK14_RD) share significant homology of 7 % identity with a Z-score of 6,6 (Akaboshi et al., 2008). The studies on these RCAR-related proteins demonstrate a ligand binding capacity for this class of proteins.

Reports on the function of different MLPs reflect the heterogeneity of this protein family. For example, some MLPs are rapidly induced after a gravity stimulus (Kimbrough et al., 2004). Chibani et al. (2006) suggested a role for other MLPs in the regulation of seed dormancy. The transcriptional regulation of some MLP members indicates a role in defense signaling. For instance, four MLPs were strongly down-regulated in the course of the clubroot disease caused by *Plasmodiophora brassicae* infection, which is features deregulated cytokinin homeostasis (Siemens et al., 2006). Jones et al. (2006) showed that the protein levels of At4g23670 were increased in response to pathogen associated molecular patterns (PAMP). Similar reactions were observed by (Devos et al., 2006) for two MLPs. The infection of Arabidopsis with tobacco mosaic virus (TMV) caused transcriptional down-regulation of MLP family proteins (Golem and Culver, 2003).

According to the paradigm of a ligand-dependent RCAR-mediated inhibition of clade A PP2Cs in ABA signaling, a Y2H approach was aimed to identify signaling modules similar to the PP2C-RCAR relay. Therefore, systemic interaction studies between a number MLPs and a subset of randomly chosen PP2C proteins from different subgroups were performed (see Fig. 38). Given the ABA-dependent stabilization of PP2C-RCAR interaction, these experiments were also performed in the presence of a number of possible ligands. Nevertheless, these analyses did not result in any positive interactions. Eventually, personal communication with A. Spena led to the confirmation of a PP2C-MLP pairing in yeast. A clade F PP2C weakly interacted with one of the Bet v 1 subunits of MLP34 (see Fig. 40). Some clade F PP2Cs were so far also reported to be involved in defense response in Arabidopsis. PIA1 and PAPP2C, two members of clade F, were shown to be negative regulators of SA-dependent signaling under biotic stress conditions (Wang et al., 2012; Widjaja et al., 2010). Also WIN2, the PP2C interacting with MLP34, is involved in defense response to *P. syringae* (Lee et al., 2008). In the end, the connection of both, clade F PP2Cs and some members of the MLP superfamily, to SA-mediated signaling, indicated a role in biotic stress response for the complex identified. SA is a major signal in the plant´s response to biotic stress. It mediates HR at the local site of infection and also systemic acquired resistance (SAR) in remote parts of the plants. Besides SA, also its derivates (e.g. methyl-SA), SA agonists (e.g. pipecolic acid) and a number of other phytohormones play important roles in

defense responses and plant immunity (Fu and Dong, 2013; Navarova et al., 2012; Ton et al., 2009; Vlot et al., 2009). Moreover, a receptor for SA itself has recently been identified (Wu et al., 2012).

In the case of WIN2-MLP34 coupling, exogenous supplementation of various phytohormones and defense-related substances did not significantly increase the interaction detected in yeast. Indeed, the nature of possible ligands ranges from small aromatic compounds or lipids to small peptides. For example, such a peptide-dependent regulation of PP2C function has recently been shown for the DNA binding phosphatase 1, DBP1 by Castello et al. (2011). This special PP2C was found to possess transcriptional regulatory activity and to be involved in virus resistance pathways (Carrasco et al., 2003; Castello et al., 2010). Furthermore the shuttling of DBP1 is connected to a 14-3-3 protein it is interacting with (Carrasco et al., 2006). In enzymatic assays, the addition of truncated MLP34 protein did not interfere with the phosphatase activity (see Fig. 42). In the case of ABA-dependent inhibition of the PP2Cs by the RCARs, there are certain co-receptor combinations that do not need the ligand for function (Hao et al., 2011). There might be combinations of clade F PP2Cs and MLPs that act in a similar fashion. Another vitally important step for the understanding of this signaling module is the identification of the appropriate ligand. This could be achieved by mass-spectroscopic analyses of immobilized WIN2-MLP34 complexes incubated with plant cell extracts (Szostkiewicz, 2010).

The results gathered in yeast and also the common involvement of PP2Cs and MLPs in defense response, implicated further studies on the function of MLPs in SA-related signaling *in vivo*. Therefore, a PR1-based reporter system to monitor SA-dependent signal transduction was established in protoplasts. The PR1 gene is upregulatetd at the later stages of SA-mediated response and reflects the onset or activation of SAR signaling (Cameron et al., 1999; Lebel et al., 1998). The effect of co-expressing MLPs or defense related PP2Cs on this particular reporter system was analyzed. Most of the MLPs tested did not cause significant changes. Nevertheless, MLPs B, C, D, F and H slightly induced pPR1::LUC reporter activity compared to the empty vector controls, in the absence or presence of SA (see Fig. 44). Preliminary experiments using other defense-related reporter systems such as pNHL10 or pPHI1 did not confirm these results (data not shown). The induction of SA-related signaling is reminiscent with the function of the RCARs in ABA response. However, the parameters of these reporter systems have to be carefully optimized to obtain a reliable read-out. Due to the fact that the general defense response is governed by a number of different hormones (SA, JA etc.) and their derivates, the reporter system to study MLP function has to be optimized.

In conclusion, the function of MLPs and clade F PP2Cs in defense signaling remains to be elucidated. However, there is a striking overlap of common components between ABA-dependent signal transduction and SA-related defense signaling. The minimal set of components in ABA signaling comprises the ABA-binding protein RCAR, the major negative regulator PP2C, downstream protein kinases and ABF TFs of the bZip class (Fujii et al., 2009; Gonzalez-Guzman et al., 2012; Joshi-Saha et al., 2011). A similar cascade of ligand binding sensors, negatively regulating PP2Cs, protein kinases and transcription factors might be present in SA-dependent defense signaling. The models depicted in Fig. 54 summarize the proposed core regulatory modules of ABA and SA signaling.

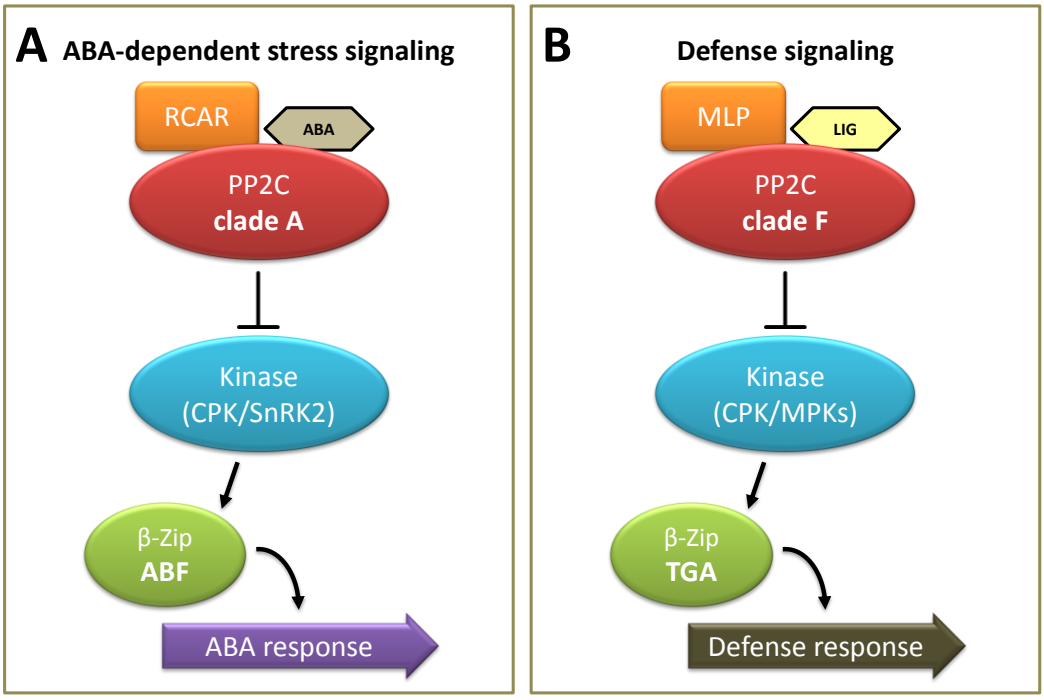


Fig. 54: Models of RCAR and MLP function. (A) The current model of ABA-related signaling: the receptor RCAR binds the ligand ABA and subsequently deactivates PP2Cs from clade A. Downstream kinases, e.g. CPKs or SnRK2s, are released from the PP2C restraint and free to phosphorylate and activate ABF TFs, which initiate transcriptional reprogramming. (B) A working model for MLP function in defense signaling: MLPs bind a defense-related ligand (LIG) and subsequently deactivate PP2Cs from clade F, which are negative regulators of defense related signal transduction. Downstream kinases, e.g. CPKs or MPKs, are released from the PP2C restraint and free to phosphorylate and activate TGA TFs involved in biotic stress response.

The central regulatory cascade for ABA signaling is activated by the perception of ABA through the RCAR proteins (Fig. 54A). This invokes the inactivation of clade A PP2Cs and in turn, an activation of downstream kinases SnRK2s, CIPKs and CPKs (Brandt et al., 2012). Finally, ABFs are phosphorylated to transcriptionally control ABA responses (Sirichandra et al., 2010). With this work, a similar pathway is proposed for defense signaling in Arabidopsis. MLPs are plausible candidates to bind

defense related ligands, such as small signaling molecules, lipids or peptides involved in defense signaling (Lytle et al., 2009). As clade F PP2Cs were shown to be involved in SA defense signaling, possible ligands could range from SA itself to microbial molecules or damage-induced aromatic substances (Bhattacharya et al., 2010). A negative regulation of SA-related traits by clade F PP2Cs has been clearly demonstrated (Lee et al., 2008; Wang et al., 2012; Widjaja et al., 2010), which is reminiscent with the situation of clade A PP2Cs in ABA signaling. However, the downstream protein kinases in SA signaling include MPKs (Asai et al., 2002; Brodersen et al., 2006; Ekengren et al., 2003) and CPKs (Boudsocq et al., 2010; Coca and San Segundo, 2010; Kanchiswamy et al., 2010), which could be positive regulators in this cascade. Eventually the transcriptional regulation is mediated by TGA TFs of the bZip family (Kesarwani et al., 2007; Mahalingam et al., 2003; Zander et al., 2010). Similar to the CPK-mediated control of ABF function, the CPKs in SA signaling might directly regulate TGA TFs to drive defense-related transcription (Blanco et al., 2009; Boudsocq et al., 2010; Coca and San Segundo, 2010).

The identification of a PP2C-MLP complex in yeast, previous reports on their function in biotic stress signaling and the effects of MLPs and PP2Cs on SA-dependent signaling *in vivo* suggest the presence of a PP2C-RCAR complex-like signaling relay also in the Arabidopsis defense response.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Reagents and Chemicals

If not stated separately, reagents and kits for molecular biology and chemicals of highest purity were used in the experiments and purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany), Roth (Carl Roth GmbH & Co KG, Karlsruhe, Germany), Qiagen (Qiagen GmbH, Hilden, Germany), Invitrogen (Life Technologies GmbH, Darmstadt, Germany), Stratagene (Stratagene GmbH, Heidelberg, Germany) or Macherey-Nagel (Macherey-Nagel GmbH & Co KG, Düren, Germany).

4.1.2 Equipment

Appliance	**Model**	**Company**
Centrifuges	Avanti J-25	Beckmann Coulter
	Type 5424	Eppendorf
	Type 5415R	Eppendorf
	Universal16	Hettich
Electrophoresis Power Supply	EPS200 / EPS301	Pharmacia Biotech
Electroporation System	Eporator	Eppendorf
Electrotransfer System	Trans-Blot semi dry	BioRad
Geldoc UV System	P91D / P93D	Mitsubishi
	Reprostar 3	Camag
	UV 312nm	Bachofer
	Doc-It	UVP
Horizontal Shaker	Rotamax 120	Heidolph
Incubators	Brutschrank ED53	WTB Binder
	Thermoshake	Gerhardt
Luminometer	Flash´n´Glow	Berthold
Magnetic Stirrer	HSC	VELP Scientifica
Microplate Reader	Synergy 2	BioTek
Microscope	Axioskop HBO50	Zeiss
Microwave	MC-9287UR	LG
NanoDrop-Photometer	NanoPhotometer 7211	Implen

PCR-Cycler	T-Gradient	Biometra
pH-Meter	pH526	WTW
Photometer	Ultrospec 2000 (UV/Visible)	Pharmacia Biotech
Scales	Microscale BP110S	Sartorius
	Labscale BP3100S	Sartorius
SDS-PAGE System	Mini Protean Tetra System	BioRad
Sterile Bench	Laminar Flow Workstation	Microflow
Thermomix	Thermomixer comfort	Eppendorf
Ultrasonifier	Sonopuls HD2070	Bandelin
Vacuum Concentrator	Membran VP	Vacuubrand
	Vacuum Concentrator	Bachofer
Vertical Shaker	RotoShake Genie	Scientific Industries
Vortex	MS3 basic	IKA

4.1.3 Organisms and cultivation

Bacterial strains

Agrobacterium tumefaciens

Strain	**Genotype**	**Resistance**	**#**	**Provider**
C58 pGV3101	*Ti-plasmid pMP90*	Rifampicin Gentamycin	845	Csaba Koncz (MPI Cologne)

Escherichia coli

Strain	**Genotype**	**Resistence**	**#**	**Source**
DH5α	*F- φ80lacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-*	-	3334	Invitrogen
XL1-blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZΔM15 Tn10 (Tetr)]*	Tetracyclin	3340	Stratagene
M15 pREP4	*Derived from strain K12, NalS strS rifS thi- lac- ara- gal+ mtl- F- recA+ uvr+ lon+ [pREP4 KanR]*	Kanamycin	3337	Qiagen

Cultivation and selection of bacteria

LB broth [g/l]

Bacto-Tryptone	10
Bacto-Yeast extract	5
NaCl	10
Agar	14 (for solid media only)

Adjust pH to 7,0 (NaOH) and autoclave.

For preparation of selective media, antibiotics are added in the appropriate concentration after cooling LB below 50 °C.

Bacteria were cultured in LB media either in shakers at 200 rpm for liquid culture or on agar plates in incubators. Temperatures were set at 30 °C for Agrobacteria or 37 °C for *E. coli* cells. For selection the medium was supplemented with the corresponding antibiotics.

Antibiotic	**Final concentration [mg/l]**	**Stock solution [mg/ml]**
Ampicillin	50-100	100 (in mQ H_2O)
Chloramphenicol	34	68 (in 100% Ethanol)
Gentamycin	25	25 (in mQ H_2O)
Kanamycin	25-50	50 (in mQ H_2O)
Rifampicin	25	25 (in 100% Ethanol)
Tetracyclin	25	50 (in mQ H_2O)

Yeast strains used in this work

AH109	*MATa, trp1-109, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA::MEL1UAS-MEL1TATA-lacZ*	#3332	from Clontech Matchmaker-system*

* Y2H system according to James et al. (1996)

Cultivation and selection of yeast

Yeast media

YPD (Yeast extract/Peptone/Dextrose) [g/l]

Yeast extract	10
Peptone	20
Agar	18 (for solid media only)

Add A.d. to 900 ml and adjust pH to 5,8 with HCl. Autoclave media and allow cooling to below 50 °C and add 100 ml of sterile 20 % glucose (dextrose) to a total volume of 1000 ml per bottle.

Synthetic Dropout (SD) minimal medium (per 1000 ml)

Macrosalts (10 x)	100 ml
Microsalts (1000 x)	1 ml
mQ H_2O	ad 800 ml
Agar	16 g (for solid media only)

Adjust pH to 5,8 with HCl. After autoclaving add 100 ml of sterile 20 % glucose and 100 ml of sterile 10 x Dropout (DO)-solution.

10 x Macrosalts for SD [g/l]

KH_2PO_4	10
$MgSO_4$ x 7 H_2O	10,24
NaCl	1
$CaCl_2$	1
$(NH_4)_2SO_4$	50

1000 x Microsalts for SD [mg/l]

Biotin	2
Ca-Pantothenic acid	400
Folic acid	20
myo-Inositol	2
Nicotinic acid	400
p-Aminobenzoic acid	200
Pyridoxine-HCl	400
Riboflavine	200
Thiamin-HCl	400
Boric Acid	500
Cu_2SO_4	40
KI	100
$FeCl_3$	200
$MnSO_4$	400
Na_2MoO_4	200
$ZnSO_4$	400

Store sterile-filtered aliquots at -20 °C

10x DO-Solution [mg/l]

L-Adenine-Hemisulfat	200
L-Arginine-HCl	200
L-Isoleucine	300
L-Lysine-HCl	300
L-Methionine	200
L-Phenylalanine	500
L-Threonine	200
L-Tyrosine	200
L-Uracil	200
L-Valine	1500
L-Histidine-HCl x H_2O	200
L-Leucine	1000
L-Tryptophane	200

For selection of transformed yeast cells solid and/or liquid SD media deficient for plasmid encoded amino-acids was used for cultivation.

Plant ecotypes and mutants

All *A. thaliana* lines used in this work are ecotype Columbia (Col-0). These plants were used as source of plant material, protoplasts, RNA and DNA as well as for stable transformation. All accessions were retrieved from the Arabidopsis Biological Resource Center (ABRC), Ohio, USA.

Plant culture

For sterile cultivation of plant material, seeds are surface sterilized and plated on MS media. To break remaining dormancy and to synchronize germination the seeds are put to 4 °C for 24 - 48 h before cultivation in the cell culture room at constant light with 60 µE m^{-2} s^{-1} and 22 °C for the time desired.

Seed sterilization

Dry Arabidopsis seeds are transferred to reaction tubes, immerged in 1 ml of the washing solution and incubated for 20 min under constant shaking at 600 rpm. After removing the supernatant the seeds are washed with 1 ml 3 % (v/v) NaOCl-solution for 2 min. To remove remaining chemicals the seeds are eventually cleaned by thorough washing with sterile mQ H_2O (5 x).

Washing solution

80 % EtOH (v/v)
0,1 % Triton X-100 (w/v)

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) (per 1000 ml)

Macrosalt-solution (10x)	100 ml
Microsalt-solution (400x)	2,5 ml
Sucrose	10 g
MES	1 g
Agar	10 g (for solid media only)

Adjust pH 5,8 (KOH) and autoclave

10 x Macrosalt-solution [g/1000 ml]

KNO_3	19,0
NH_4NO_3	16,5
$MgSO_4$ x 7 H_2O	3,7
CaCl2 x 2 H_2O	3,3
KH_2PO_4	1,7

Autoclave for storage

400 x Microsalt-solution [g/1000 ml]

Na_2EDTA	14,6
$FeSO_4$ x 7 H_2O	11,2
$MnSO_4$ x 4 H_2O	4,0
H_3BO_3	1,2
$ZnSO_4$ x 4 H_2O	0,8
KI	0,3
$CoCl_2$ x 6 H_2O	0,01
$CuSO_4$ x 5 H_2O	0,01
Na_2MoO_4 x 2 H_2O	0,1

Store at -20 °C

4.1.4 Plasmids and Oligonucleotides

All the constructs generated and used in this work are listed in the appendix (see 6.1). The clones carrying the respective plasmids are stored at -80 °C in the Institute´s strain collection labeled with the numbers indicated in the list. All the oligonucleotides created and used in this work are listed in the appendix (see 6.2), stored at -20 °C in the Institute´s primer collection and labeled with the numbers indicated in the list.

4.2 Methods

This work was performed according to the standard procedures and protocols published as laboratory manual by Sambrook and Russell (2001). Modifications and adaptations of single protocols and experiments are separately mentioned.

4.2.1 Plasmid Preparation

Plasmid Mini Preparation

The bacterial clones of interest are inoculated in 5 ml LB-media containing the appropriate antibiotic. 2-4 ml of the cell suspension are pelleted at 15000 x g for 3 min and resuspended in 100 µl of solution E1. For lysis 200 µl of solution E2 are added. After cautious mixing 150 µl of E3 solution are added to precipitate protein and cellular debris. The DNA containing supernatant gained by centrifugation at 15000 x g for 3 min is transferred into a new reaction tube containing 900 µl of cold EtOH. The DNA precipitating is harvested by centrifugation for 20 min at maximum speed and washed with 70 % EtOH. The dried pellet of plasmid DNA is resuspended in 30 µl mQ water and used for further analyses.

Solution 1	Solution 2 (Lysis Buffer)	Solution 3
50 mM Glucose 25 mM Tris HCl (pH 8,0) 10 mM EDTA	0,2 M NaOH 1 % SDS	3 M NaAc (pH 4,8-5,2)

Alternative mini-prep volume calculation (after SMS): $x * 2 - 50 = (x + 2x) : 2$

Plasmid Midi Preparation

To generate higher amounts of purified plasmid DNA the JETSTAR Plasmid Midiprep Kit (Genomed, Löhne, Germany) is used. Solutions and columns are used according to the protocols in the kit. Shortly, a 100 ml LB O/N culture per construct is centrifuged at 5000 x g for 15 min. The pellet is resuspended, lysed and purified according to the procedures given. Column bound DNA is washed, eluted and precipitated in isopropanol. The purified DNA is pelleted, dried and subsequently dissolved in 200-400 µl mQ H_2O.

4.2.2 Polymerase Chain Reaction (PCR)

Standard mixture

Component	Final conentration	Volume [µl]
Template DNA	0,1 - 20 ng	1
Buffer (including 25 mM $MgCl_2$) [5x or 10x]	1 x (2,5 mM $MgCl_2$)	2-4
dNTP-Mix [10 mM]	1 mM	2
Primer forward (stock 100 µM)	1 µM	0,2
Primer reverse (stock 100 µM)	1 µM	0,2
Polymerase [2-5 U/µl]	0,01-0,25 U/µl	0,05-0,2 µl
mQ H_2O		ad 20 µl

Depending on template, primers and polymerase the reaction was supplied with additives such as additional $MgCl_2$ (up to 10 mM) or DMSO [2%] to improve reaction efficiency, when required.

Cycling

Step	Duration [min]	Temperature [°C]	#
Initial Denaturing	2-5*	95	1
Denaturing	0,25-1*	95	30-35
Annealing	0,25-1*	48-65**	
Elongation	0,5-10*	68-72*	
Final Elongation	3-7*	68-72*	1

* depending on the polymerase used
** depending on the Tm of the primer pair

Polymerases used in this work and specifications

Polyermase	Proof-Reading	Buffer	Amplification [kb/min]	Company
GoTaq	-	5x	1	Promega
Vent	+	10x	0,5	NEB
Pwo	+	10x	0,5	PeqLab
Pfu	+	10x	0,5	Promega
UltraPfu	+	10x	1 - 2	Stratagene
Phusion	+	10x	2 - 3	Thermo Scientific

4.2.3 Agarose Gel Electrophoresis

For size separation and purification of DNA fragments 1 x TAE buffer was supplemented with 1 % (w/v) agarose powder. The mixture is boiled in the microwave oven until the powder is completely dissolved. After cooling the liquid gel to below 60 °C ethidium bromide (EtBr) stock solution was added 1:10000 and the gels desired were poured. After polymerization the DNA samples provided with loading dye to 1 x final concentration were applied and electrophoresis was run in 1 x TAE running buffer for usually 30 min at 200 V and 400 mA.

50 x TAE Buffer (per 1000 ml)

Tris-Base	242 g
Acetic Acid	57,1 ml
0,5 M EDTA (pH 8,0)	100 ml

6x Loading Dye

Glycerol	50 % (v/v)
Orange G	0,25 % (w/v)
EDTA (pH 8,0)	1 mM

EtBr stock solution

10 mg EtBr in 1 ml mQ H_2O

Running buffer

1 x TAE with EtBr (1:10000)

Gel extraction

DNA fragments of interest were excised as gel slice from the agarose gels with a scalpel and then purified using the PeqLab Gel Extraction Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). Solutions were applied according to the kit´s manual. Nucleic acid exchange columns were regenerated and reused several times. For elution 20 - 50 µl mQ H_2O were used.

Regeneration of nucleic acid extraction columns (NAEC)

Commercially available spin columns can easily be prepared for recycling (Siddappa et al., 2007). After initial use, DNA remains and cell debris are removed from NAEC by thorough washing with water and 0,1 M HCl. The columns can be stored in the acidic solution for months. Before reuse the columns have to be again thoroughly washed five times with mQ H_2O and once with 1 x TE. The dry columns can then be loaded again with new samples without any contamination problems.

Quantification of DNA content and size determination

The concentration of DNA solutions is determined either by measurement of an aliquot in a nanodrop photometer or by comparative estimation of EtBr-stained agarose gel bands after electrophoresis. The size of DNA bands was compared to standard DNA size markers.

Marker	Company
GeneRuler 1 kb DNA Ladder	Thermo Scientific
GeneRuler 100 bp DNA Ladder	Thermo Scientific
Lambda DNA / HindIII Marker	Thermo Scientific

4.2.4 Cloning Procedures

Digest of DNA fragments and plasmids

For cloning and plasmid analysis restriction endonucleases from Thermo Scientific (formerly MBI Fermentas, St. Leon-Rot, Germany) and NEB (New England Biolabs, Ipswich, USA) were used. Usually for 1 µg of DNA the standard 10 x buffer was diluted and the restriction enzyme(s) [0,5 - 1 U] were added for digestion at 37 °C for 1 - 2 h. Plasmid restriction patterns of cloned constructs were analyzed running an agarose gel. PCR products and plasmids for cloning were purified on columns or by gel extraction before ligation.

Ligation

Inserts and linearized vectors were ligated after digestion and purification in a 5 - 10:1 ratio. The standard reaction contained 30 - 50 ng of vector backbone, an appropriate amount of insert, 1 x ligase buffer and 0,5 µl T4-DNA-Ligase filled to a total volume 10 µl with mQ H_2O. The sample was incubated for 0,5 - 2 h at RT before being used for transformation of competent cells.

4.2.5 Transformation Procedures

Competent cells

For the preparation of chemo-competent cells 250 ml of *E. coli* culture are grown at 18 °C in medium A to an OD_{600} of 0,6. Cells are cooled on ice for 10 min prior to harvest at 4°C for 15 min at 2500 x g. Pellet is resuspended in 80 ml cooled TB medium. After supplementation of 0,7 ml DMSO cells are incubated for 10 min on ice. Aliquots of 200 µl cell suspension are frozen in liquid N_2 and stored at -80 °C for later use.

Medium A		**TB-Medium**	
Peptone	20 g/l	PIPES (pH 6,4)	10 mM
Yeast extract	5 g/l	$MnCl_2$	55 mM
NaCl	10 mM	$CaCl_2$	15 mM
KCl	2,5 mM	KCl	250 mM
$MgCl_2$	10 mM		
$MgSO_4$	10 mM		

4.2.6 Site-directed mutagenesis (SDM)

The replacement, deletion of insertion of amino acid residues within a protein is a useful approach to study functional importance. SDM procedures were performed based on the protocols of the Stratagene Site-directed Mutagenesis Kit (Agilent Technologies, Santa Clara, USA), Zheng et al. (2004) and Liu and Naismith (2008). In short, oligonucleotide primers with an approximate length of 30 bp carrying the desired modifications (substitutions, deletions or insertions) were designed. The primers were used in a PCR with UltraPfu proofreading polymerase (Stratagene) to generate single stranded copies of the template plasmid. Maternal plasmids are subsequently removed by DpnI digest of methylated DNA. PCR amplification products are not methylated and hence not digested. Remaining single stranded DNA will anneal complementarily. Finally *E. coli* strain XL-1 blue (Stratagene), capable of repairing single strand breaks in plasmids, is transformed with the DpnI-digested PCR product. The clones obtained are checked for the desired modification by restriction analysis and sequencing.

Standard PCR reaction for SDM

4 µl dNTPs
4 µl Buffer UltraPfu [10x]
1 µl primer fwd
1 µl primer rev
0,2 µl UltraPfu polymerase (Stratagene)
1 µl template plasmid [1-25 ng/µl]
ad 40 µl mQ H_2O

4.2.7 Yeast transformation

Saccharomyces cerevisiae transformation is performed using the LiAc transformation based on the techniques published by Gietz and Woods (Gietz and Woods, 2002). Single yeast colonies are used to inoculate a 5 ml preculture in SD media supplemented with the corresponding amino acids and grown O/N. An aliquot of 1 ml is used to inoculate a 50 ml culture in YPD full medium. When reaching an optimal OD_{600} of 0,6-0,8 cells are harvested via centrifugation for 5 min at 1000 x g. After washing the cells in 10 ml sterile mQ water, the pellet is resuspended in 1 ml of 1 x LiAc [100 mM] and

transferred into a new reaction tube. Cells are again pelleted by 15 sec short spinning and dissolved in 400 µl 1 x LiAc. For one transformation 50 µl of this solution are used and supplemented with:

240 µl PEG4000 (50 % w/v)
36 µl 10 x LiAc [1 M]
10 µl Carrier DNA (10 µg/µl)
1 µl DNA [usually 100 – 500 ng/µl]
73 µl mQ water

Transformation mixtures are resuspended and incubated for 40 min at 42 °C. After heatshocking the cells were harvested by centrifugation for 15 sec short spin and resupended in 100 µl mQ water for subsequent plating on selective media. Positive clones are checked for presence of the plasmids by PCR using DNA from yeast plasmid mini prep as a template.

Carrier DNA

Single stranded DNA derived from salmon testes is dissolved 10 mg/ml in 1x TE buffer (pH 7,5), stirred for 3 h and sonopulsed twice at max. power for 30 sec. After this 1/10 vol. NaAc [3M] is added to this solution before DNA is precipitated by mixing with 2,5 vol. of EtOH. The precipitated DNA is pelleted by centrifugation for 10 min at max. speed and then dried. After resolving the DNA in 1 vol. TE buffer it is denaturated at 95 °C for 20 min and frozen for later use. The aliquots of carrier DNA to be used for transformation are again boiled at 95 °C for 5 min and then cooled on ice prior to use.

4.2.8 Yeast mini plasmid preparation

For the extraction of yeast DNA 4-10 ml of the culture are harvested at 2000 x g for 2 min and washed with 1 ml mQ H_2O. The pellet was resuspended in 500 µl lysis buffer. A pinch of glass beads is added and the mixture is vigorously vortexed to lyse the cells. After sedimentation of the beads on ice, the supernatant is transferred to a new reaction tube. 750 µl of 7 M ammonium acetate (pH 7,0) are added and the tube is incubated for 5 min in 65 °C. Then the extract is put on ice for 5 min before adding 500 µl of pure chloroform. The two phases appearing are well mixed and then centrifuged for 2 min at 15000 x g. The supernatant is again transferred into a new reaction tube. DNA is precipitated by addition of 1 ml of isopropanol and incubation at RT for 5 min. The DNA is pelleted for 5 min at 15000 x g, washed with 70 % EtOH and centrifuged for 5 min at 15000 x g. Finally the pellet is dried and resuspended in 30 - 50 µl mQ H_2O. The DNA was used for corresponding experiments.

Lysis Buffer

Tris/HCl pH 8,0	100 mM
EDTA	50 mM
SDS	1 %

4.2.9 Yeast-two-hybrid (Y2H) interaction analyses

The *S. cerevisiae* strain AH109 is optimized for Y2H analyses in the Matchmaker-system (James et al., 1996). It carries several reporters suitable for evaluation of protein-protein interaction. For this work three differentially activated reporters were used.

Histidine- or Adenine-autotrophy growth assays (Y2/3H-spotting)

Singe colonies were used to inoculate 1 ml of selective SD medium. Culture was grown O/N before addition of 4 ml YPD. The OD_{600} was measured (optimally between 0,5 and 0,8) and dilution volumes were calculated. The cell number of AH109 strains at OD_{600} = 1 equals 5 x 10^7/ml. A dilution series for 1000, 100 and 10 cells per spot was pipetted for each sample according to corresponding cell density. Aliquots (usually 10 µl) of the single dilutions were spotted on different media. -L/-W plates are selective for successful dual transformation of AD and BD plasmids. -L/-W/-H, -L/-W/-A, and -L/-W/-A/-H plates select for interaction of the proteins tested with increasing stringency. Supplementation of the media with 3AT can inhibit His-reporter leakiness. For the Y3H analyses additional plates either lacking methionine (Met) or supplemented with 0,15 or 1 mM Met were produced. Met represses expression of the third component upstream of the Met2 promoter in the second multiple cloning site of the pBridge vector in a concentration-dependent manner (Tirode et al., 1997).

His-autotrophy Y2H liquid culture growth assay (YLGA)

Singe colonies were used to inoculate 1 ml of selective SD medium. Culture was grown O/N before addition of 4 ml YPD. The OD_{600} was measured (optimally between 0,5 and 0,8) and dilution volumes were calculated. The yeast cells were cultured in sterile 96 well cell culture plates (Cellstar plates, purchased from Greiner Bio-One GmbH, Frickenhausen, Germany) under constant shaking at 200 rpm. For inoculation of 200 µl selective SD media 2 x 10^4 cells of each yeast line were used. Growth in selective media was monitored as change in OD_{600} over time compared to non-inoculated selective media used as blank.

β-galactosidase activity assay (β-Gal assay)

Singe colonies were used to inoculate 1 ml of selective SD. Culture was grown O/N before addition of 4 ml YPD. At an OD_{600} from 0,6-0,8 aliquots of 1 ml culture were harvested by short spinning for 15 sec. The supernatant is replaced with 0,5 ml buffer Z. After washing the cells are again pelleted and resuspended in 100 µl buffer Z. The cells are lysed by repeated freeze-thaw-cycles (5x) in liquid nitrogen. After lysis 700 µl of Z+βME were added to the extract. The β-Gal reaction is started by adding 160 µl ONPG solution. Samples are incubated at 30 °C until first yellow coloring is visible. Then the reactions are stopped by the addition of 400 µl of 1 M Na_2CO_3. The time from providing the substrate till stopping the reaction is recorded. For final measurement cellular debris is removed by centrifugation at 10000 x g for 10 min. The supernatant is transferred to cuvettes. OD_{420} is determined against a blank of accordingly treated control samples without cells. β-gal activity is calculated in Miller units according to the formula

$$X=1000*OD_{420}/(t*OD_{600})$$

t= time of sample incubation after addition of substrate

Buffer Z (pH 7,0)

Na_2HPO_4 x 2 H_2O	60 mM
NaH_2PO_4 x H_2O	40 mM
KCl	10 mM
$MgSO_4$ x 7 H_2O	1 mM

Autoclave

Z+βME 270 µl β-mercapto-ethanol in 100 ml buffer Z

ONPG solution 4 mg/ml dissolved in buffer Z

4.2.10 Floral Dip

For stable transformation of Arabidopsis plants the floral dip procedures according to Harrison et al. (2006) and Zhang et al. (2006) are used. Recipient plants were grown for 12 - 14 d until inflorescences were present but not yet opened. The *A. tumefaciens* strains carrying the desired plasmid are cultivated O/N in 200 ml LB media, pelleted for 10 min at 5000 x g and resuspended in 300 ml infiltration medium. Plants are submerged in the bacterial suspension for 30 sec assuring complete wetting of the stems and inflorescences. After dipping plants are carefully tied and supplemented with 1 x macrosalt-solution. To increase transformation rates, the procedure was usually repeated once, 3 days after the first dipping.

Infiltration medium

5 % sucrose
0,5 % VAC-IN-STUFF Silvet (L-77)
(from Lehle Seeds, Round Rock TX, USA)

Macrosalt-solution

(see MS media)

Binary vectors for Agrobacterium mediated plant transformation

The transgene is transmitted via agrobacterial infection into plant cells. The vector system is based on the pBI121 binary vector (Jefferson, 1987) carrying the gene of interest in the T-DNA flanked by left and right borders. The transgenes in this case consist of a promoter element, the sequence of interest, epitope tags and a termination sequence. Based on the system established by Hoffmann (2001), the AscI flanked cassette including the transgene is cut out of the pSK derived donor vectors by AscI restriction for 2 h, and inserted into the AscI linearized pBI121 AscI fragment. The derived clones are checked by PCR or digest for proper insertion. The plasmid is then used for the transformation of the electro-competent Agrobacterium strain #845 (see 6.1).

4.2.11 Isolation and transfection of protoplasts

The general procedure of harvesting and transfecting mesophyll protoplasts was adapted from Yoo et al. (2007), Ma et al. (2009) and Himmelbach et al. (2002). Shortly, rosette leaves of Arabidopsis plants are carefully separated from the plant and transferred into a petri dish containing 10 - 12 ml of enzyme solution. Leaves are precautiously submerged and digested at room temperature for 3 - 4 h on a RotoShake vertical shaker at 30 rpm. The suspension of disintegrated leaves is then filtered through a nylon mesh (~150 µm wide).

Before transformation the remaining enzyme solution has to be removed. Therefore, 8 ml of WIMK solution is added to the suspension. After centriguation at 60 x g for 3 min the pellet is carefully resuspended in 4 ml WIMK. After another centrifugation step the pellet is resuspended in an appropriate volume of MM solution to generate a solution with a concentration of 0,5 - 1 x 10^6 protoplasts/ml. To estimate the portion of living protoplasts, staining with Fluorescein-diacetate (FDA) was performed before counting in the hematocytometer.

The suspension is then cooled to 4 °C for 30 min to improve transformation efficiency. For the transfection, a mixture of the plasmids to be introduced to the cells is provided in reaction tubes.

Standard mixture of plasmids for reporter assay

5 µg pRD29B::LUC reporter plasmid (strain #3041)
3 µg 35S::GUS reporter plasmid (strain #883)
x µg effector plasmid / empty vector DNA
15 µl Mannitol [0,8 M]
ad 30 µl mQ H_2O

In the samples not supplied with any effector DNA, a corresponding amount of empty vector plasmid is used to compensate for carrier effects. Aliquots of the harvested and washed protoplasts are then transferred to the reaction tubes containing the reporter-effector DNA premix.

To this mixture of DNA and protoplasts 130 µl of PEG solution are added and inverted 5 times to assure proper mixing of the solutions. After incubation for 5 min, the reaction is stopped by adding 750 µl WIMK and inverting the sample twice. The protoplasts are pelleted using a table-top centrifuge at 200 g for 2 min. The supernatant is removed and the cells are resuspended in 300 µl WIMK. After one more cycle of careful mixing and spinning the protoplasts are resuspended in 200 µl WIMK. The samples are then split in two 100 µl aliquots incubated with or without the inductive substances, such as ABA or SA. The standard reporter DNA mixture used for the new SA-dependent reporter system contains 7 µg of pPR1::LUC reporter instead of 5 µg of pRD29::LUC plasmid. The rest of the assay remains the same as for ABA-dependent signaling. The protoplast samples are incubated for usually 18 h on a Rotamax horizontal shaker at 50 rpm.

Reporter assay

The 35S promoter driven GUS reporter is used to assess transformation efficiency and to normalize the LUC activity of single samples. It is measured as fluorescence signal derived from and correlating to the β-glucoronidase acitivity in the sample. 50 µl of properly resuspended protoplasts are mixed with 100 µl of 1,5 x MUG-solution and monitored for 12 min in the BioTek Synergy 2 plate reader. GUS activity is calculated and given as relative fluorescence units (RFU)/sec.

The second reporter contains the promotor element of the ABA-responsive RD29B gene. If ABA signaling is activated, the luciferase gene controlled by the promoter is expressed at high levels. Thus, ABA-dependent activation of the reporter pRD29B::LUC can be measured. For the measurement 50 µl of the protoplast samples are directly transferred into luminometer round bottom tubes. The sample tubes are automatically processed and 100 µl of LAR substrate are automatically added to start the luminescence reaction. Total LUC activity is given as relative luminescence units (RLU) and allows calculation of RLU/sec. The relative activity of LUC to GUS reflects the activation of ABA-dependent signaling.

Solutions

WIMK
500 mM Mannit 5 mM MES pH 5,8 (TRIS)

Magnesium-Mannitol (MM) solution
400 mM Mannit 15 mM $MgCl_2$ 5 mM MES pH 5,6 (KOH)

PEG solution
40 % (w/v) PEG 4000 300 mM $CaCl_2$ 5 mM MES pH 5,6 (KOH)

MUG buffer
50 mM Na_2HPO_4/NaH_2PO_4 (pH 7,0) 10 mM Na_2EDTA 0,1 % (w/v) Triton X-100 1 mM DTT 0,2 mM 4-Methylumbelliferyl-β-D-Glucuronid (MUG)

Enzyme solution
1 % Cellulase (w/v) 0,25% Macerozyme (w/v) 0,5 mM PMSF 400 mM Mannit 8 mM $CaCl_2$ 5 mM MES 0,1% BSA (w/v) pH 5,6 (KOH)

LAR buffer
1 % (w/v) Triton X-100 0,5 mM EDTA 30 mM DTT 0,5 mM ATP 2,7 mM $MgSO_4$ 1 mM $(MgCO_3)_4Mg(OH)_2$ 250 µM Coenzyme A 500 µM Luciferin 20 mM Tricine/NaOH (pH 7,8)

4.2.12 Protoplast expression vectors

To achieve ectopic expression of PP2C and RCAR effector proteins an adequate protoplast vector was generated. Based on a pBluescript II SK (Alting-Mees et al., 1992), Hoffmann (2001) constructed such a plant overexpression vector. The construct pSK 35S EYFP (#720) from the institute´s strain collection was chosen as a suitable template and checked by control digests and sequencing. Ectopic expression of the gene of interest is driven by the highly active Cauliflower Mosaic Virus (CaMV)-derived 35S promoter (Benfey and Chua, 1990; Odell et al., 1985). The expression is aborted by the Nopalin synthethase (Nos) terminator sequence. The new construct should allow for an easy transfer of PP2C and RCAR sequences from the Y2H vector system to the protoplast expression vector. Therefore, a new multiple cloning site (MCS), compatible to pGAD/pBridge vectors, was introduced. Before this undesired unique EcoRI and SalI sites on the backbone of the original #720 construct had to be removed. After an EcoRI/SalI double digest, the backbone fragment was blunted using Vent polymerase and subsequently re-ligated using T4 DNA ligase. Eventually the EYFP fragment from the

original vector was removed by BamHI/SacI double digest. A suitable pair of antiparallel DNA oligonucleotides were purchased from MWG (Eurofins MWG Operon, Ebersberg, Germany) and ligated to the vector fragment. The sequence of the inserted oligos was designed to be compatible to the MCS of the pGAD and pBridge vectors used in the Y2H analyses.

Genes of interest could easily be transferred from the present Y2H constructs to protoplast vectors by digest and re-ligation of fragments. A graphical map of the new vector for protoplast transient expression studies is presented in Fig. 55A. The MCS of strain #4065 is compatible to the pGAD/pBridge MCS.

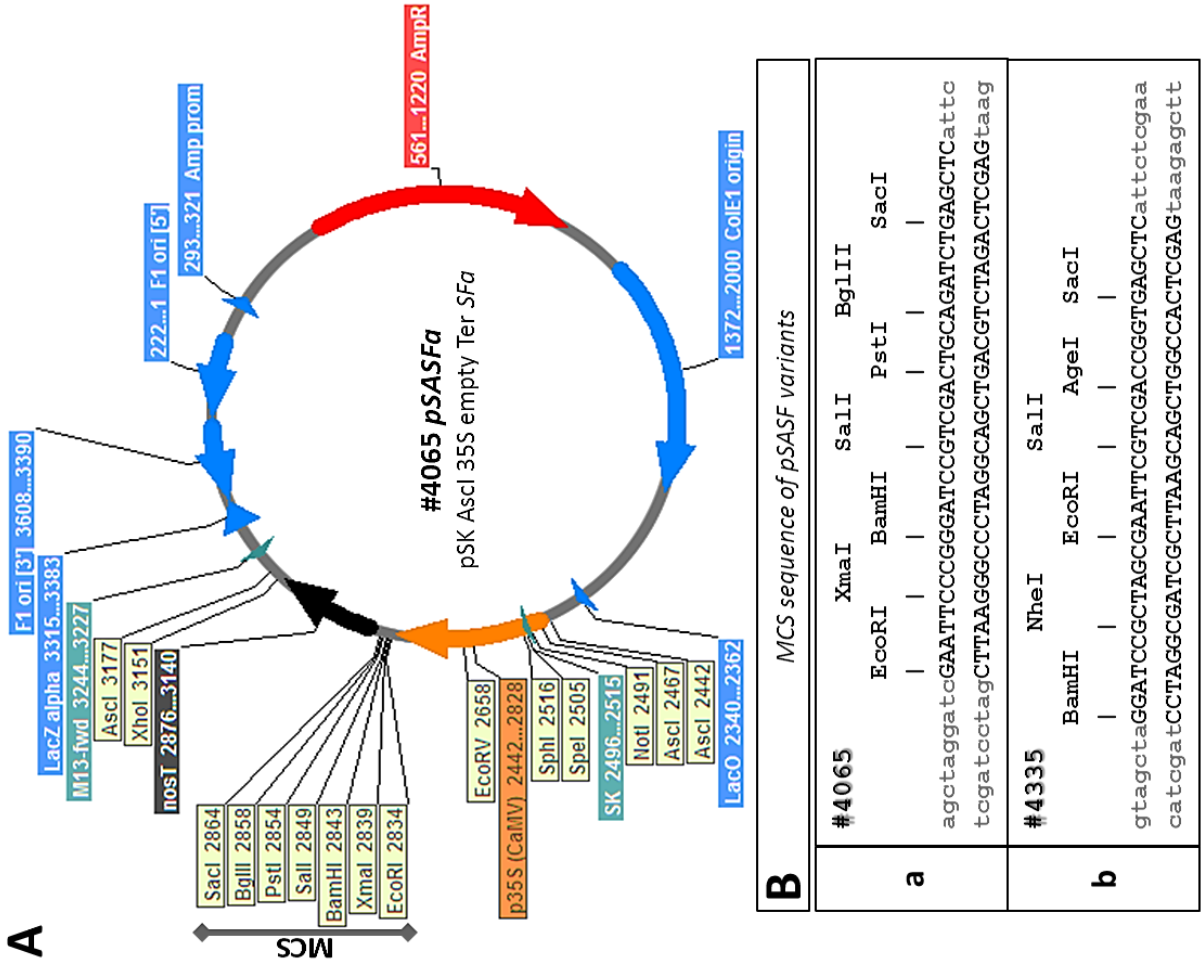


Fig. 55: Vector constructs used for transient expression in protoplasts. A pSK derivate was modified for compatibility with Y2H vectors (see text for details). (A) The graphical plasmid map shows pSASFa with ampicillin resistance (AmpR). Expression is driven by a 35S promoter (orange arrow) and stopped by Nos terminator (black arrow). In between a new MCS for insertion of the gene of interest was introduced. (B) Two variants of MCS were inserted via oligonucleotide spacer ligation. The variants pSASFa (strainlist #4065) and pSASFb (strainlist #4335) carry seven and six unique sites in the MCS, respectively.

Moreover, the expression cassette containing the gene of interest flanked by the 35S promoter and the Nos terminator can be excised by AscI single enzyme digest. This AscI cassette is compatible to a

pBI121 binary vector based system (Jefferson, 1987) established at the institute by Hoffmann (2001). Binary vectors are used for *Agrobacterium tumefaciens*-mediated plant transformation (Chen et al., 2003) (see 4.2.10 for transformation details). Another second variant of the pSASF protoplast vector was designed and generated for compatibility with pGAD GH Y2H vectors, featuring different restriction sites (Fig. 55B b).

4.2.13 Preparation of plant extract

Hydrophobic substances of low molecular weight were extracted from Col cell suspension culture according to the procedures from Szostkiewicz (2010). In short, 7 d old plant cell culture material was harvested by suction filtration. The pellet was frozen in liquid nitrogen and thawed for cell lysis. Approximately 200 g material were dissolved in 100 ml mQ H_2O adjusted to pH 3,0 with 0,25 % formic acid. After stirring for 2 h at 4 °C cellular debris was removed by centrifugation at 8000 x g for 15 min and subsequent sterile filtration. The cell free extract was then loaded on Bakerbond SPE C_{18} columns (J.T. Baker, Deventer, Netherlands). Columns were equilibrated with 6 ml of 100% methanol and washed twice with 6 ml of 0,25 % formic acid before loading. After application of ~200 ml extract per column, the columns were again washed with 6 ml 0,25 % formic acid. Column bound molecules were eluted with 6 ml methanol. Afterwards, the solvent was removed by centrifugation in a vacuum concentrator. Concentrated eluate was resolved in mQ water and pH was adjusted to 6,0 for subsequent assays.

4.2.14 *In vitro* protein studies

Expression of recombinant protein in E. coli

Bacterial strains carrying pQE-70 protein expression vectors were pre-cultured in 20 ml selection medium O/N before inoculation of 400 ml LB. Protein expression was induced in bacterial cultures grown at 37 °C to OD_{600} of 0,5 with Isopropyl-β-D-thiogalactopyranosid (IPTG). IPTG concentrations for induction of bacterial translation were optimized and set to 0,5 mM for 3 h for tMLP34 and to 1 mM for 3 h for WIN2. Thereafter, cells were harvested by centrifugation for 20 min at 4000 x g and used directly for purification or frozen at -20 °C for later processing.

Protein purification

Harvested cells were incubated with 5 ml lysisbuffer including lysozyme [1 mg/ml] on ice for 30 min. The suspension was then sonopulsed for 5 x 10 s (5 cycles at 80 % power). Debris was pelleted at 4 °C for 30 min at 30000 x g. The cleared supernatant containing the His-tagged proteins was purified using Protino 2000 kit from Macherey-Nagel. Samples were loaded on equilibrated Ni-TED columns. Washing and elution steps were performed according to the instructions in the kit´s manual. For analytical experiments the column purified proteins were dialyzed using VISKING 14 kDa cut-off dialysis tubing 37/32 from SERVA (SERVA GmbH, Heidelberg, Germany). The samples were dialyzed twice against fresh buffers at 4 °C O/N.

Lysis Buffer	**Elution Buffer**
50 mM NaH_2PO_4 300 mM NaCl 5 mM Imidazole pH 8,0	50 mM NaH_2PO_4 300 mM NaCl 250 mM Imidazole pH 8,0

Washing Buffer	**Dialysis Buffer**
50 mM NaH_2PO_4 300 mM NaCl 20 mM Imidazole pH 8,0	100 mM Tris HCl 100 mM NaCl 2 mM Dithiothreitol (DTT) pH 7,9

SDS-PAGE

SDS-PAGE was performed using a 15 % separating gel and a 4 % stacking gel. Protein samples before loading on a gel were mixed with 2x loading or 5x SDS sample buffer and denaturated at 95 °C for 5 min. The gel was run at 120 - 200 V using the BioRad-PAGE-system until the blue dye has reached the bottom of the gel.

	Separating gel (15%)	*Stacking gel (4%)*
Acrylamid solution	5,0 ml	390 µl
mQ H_2O	2,3 ml	2,3 ml
Buffer (sep/stack)	2,5 ml	255 µl
10 % SDS	100 µl	30 µl
TEMED	5 µl	3 µl
10 % APS	50 µl	15 µl
Total volume	10 ml	3 ml

Reagents	**SDS Running Buffer (1x):**
Acrylamid solution: 30 % (w/v) Acrylamid / 0,8 % Bisacrylamid Buffer for separating gel: 1,5 M Tris HCl, pH 8,8 Buffer for stacking gel: 0,5 M Tris HCl, pH 6,8 SDS solution: 10 % (w/v) Ammonium persulfate (APS) solution: 10 % (w/v) TEMED: pure	25 mM Tris base 192 mM Glycine 0,1 % SDS

2 x Loading Buffer	**5 x SDS Sample Buffer**
90 mM Tris HCl (pH 6,8) 20 % Glycerol 2 % SDS 0.02 % Bromophenol blue 100 mM DTT	125 mM Tris HCl (pH 6,8) 25 % Glycerol 5 % SDS 5 % β-mercapto-ethanol (βME) 0,05 % Bromophenol blue

Marker	**Company**
PageRuler Plus Prestained Protein Ladder	Thermo Scientific

Coomassie Blue staining

In this study rapid Coomassie Blue staining procedures were used (Kurien and Scofield, 1998). The SDS-PAGE gels immersed in the staining solution were heated in 600 W output microwave oven for 2 min. Afterwards the gels were cooled at RT for 5 min with gentle shaking. After removing the staining solution, the gels were placed in destaining solution and heated at 600 W for 1 min. The gels were then allowed to cool at RT for 5 minutes with gentle shaking. At this point, protein bands containing at least 5 ng of protein could be observed.

Staining Solution	**Destaining Solution**
0,05 % Coomassie R-250 25 % Isopropanol 10 % Acetic acid	10 % Acetic acid

Protein concentrations were determined after Coomassie blue staining by comparison with defined amounts of bovine serum albumin (BSA) loaded as reference (Ramagli and Rodriguez, 1985).

Phosphatase activity assay

The activity of a phosphatase is determined by measuring the fluorescence of the catalyzed substrate 4-methyl-umbelliferylphosphate (MUP), which is proportional to the amount of active enzyme (Meinhard and Grill, 2001). Before the measurement the phosphatase mixed with interacting proteins and/or ligands was preincubated for 20 min and the 2 x substrate supplemented with freshly prepared 0,3 mM $MnCl_2$ was preincubated for 5 min, both at 35 °C. To start the reaction equal volumes of phosphatase premix and substrate premix are pipetted and mixed in a 96 well plate. The reaction is immediately transferred to the BioTek Synergy 2 plate reader. Kinetics of substrate conversion were determined with excitation at 360 nm and emission at 460 nm over 10 min to calculate mean V of the reaction and are given in relative fluorescence units (RFU)/sec.

Stock solutions	**2 x Substrate**
1 M $MnCl_2$ x 4 H_2O 1 M Tris/HCl pH 7,9 5 M NaCl 0,1 M MUP 1 M DTT	0,1 M Tris/HCl pH 7,9 0,1 M NaCl 0,3 mM $MnCl_2$ 5 mM MUP
8 x Buffer for preincubation	**Preincubation mix (for 100 µl)**
1 ml Tris/HCl 0,2 ml NaCl 50 µl DTT	12,5 µl 8 x Buffer 1,5 µl of freshly prepared $MnCl_2$ solution [20 mM] PP2C (usually 500 ng) Other proteins (x ng) mQ H_2O ad 100 µl

4.2.15 *In silico* analyses

Sequence information was retrieved from "The Arabidopsis Information Ressource" (TAIR) database (Huala et al., 2001). Alignments of protein and nucleotide sequences were performed using Clustal Omega (Sievers et al., 2011), Multalin (Corpet, 1988) and PRALINE (Pirovano et al., 2008) algorithms. Protein structures were deduced from the data available at the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB) (online at http://www.rcsb.org/pdb/home/home.do) using PyMol software for visualization (Schrodinger, 2010). Structural predictions and impositions were performed using HHpred and Modeller online toolkits of the Max Planck Institute for Bioinformatics in Tuebingen (Remmert et al., 2012; Soding, 2005; Soding et al., 2005). The evolutionary analyses presented in this work were generated with the MEGA5.1 software package (Tamura et al., 2011). The construction of virtual constructs, cloning strategies and primer design were performed with Serial Cloner software by Franck Perez, Serial Basics (available at http://serialbasics.free.fr/Serial _Cloner.html).

5 REFERENCES

Addicott, F.T., and Lyon, J.L. (1969). Physiology of Abscisic Acid and Related Substances. Annual review of plant physiology and plant molecular biology *20*, 139-164.

Addicott, F.T., Lyon, J.L., Ohkuma, K., Thiessen, W.E., Carns, H.R., Smith, O.E., Cornforth, J.W., Milborrow, B.V., Ryback, G., and Wareing, P.F. (1968). Abscisic acid: a new name for abscisin II (dormin). Science *159*, 1493.

Adie, B.A., Perez-Perez, J., Perez-Perez, M.M., Godoy, M., Sanchez-Serrano, J.J., Schmelz, E.A., and Solano, R. (2007). ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in Arabidopsis. The Plant cell *19*, 1665-1681.

Akaboshi, M., Hashimoto, H., Ishida, H., Saijo, S., Koizumi, N., Sato, M., and Shimizu, T. (2008). The crystal structure of plant-specific calcium-binding protein AtCBL2 in complex with the regulatory domain of AtCIPK14. Journal of molecular biology *377*, 246-257.

Al-Kaisi, M.M., Elmore, R.W., Guzman, J.G., Hanna, H.M., Hart, C.E., Helmers, M.J., Hodgson, E.W., Lenssen, A.W., Mallarino, A.P., Robertson, A.E*., et al.* (2013). Drought impact on crop production and the soil environment: 2012 experiences from Iowa. Journal of Soil and Water Conservation *68*, 19A-24A.

Albrecht, V., Ritz, O., Linder, S., Harter, K., and Kudla, J. (2001). The NAF domain defines a novel protein-protein interaction module conserved in Ca2+-regulated kinases. The EMBO journal *20*, 1051-1063.

Alting-Mees, M.A., Sorge, J.A., and Short, J.M. (1992). pBluescriptII: multifunctional cloning and mapping vectors. Methods Enzymol *216*, 483-495.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. Journal of molecular biology *215*, 403-410.

An, C., and Mou, Z. (2011). Salicylic acid and its function in plant immunity. Journal of integrative plant biology *53*, 412-428.

Anderson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehlert, C., Maclean, D.J., Ebert, P.R., and Kazan, K. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. The Plant cell *16*, 3460-3479.

Antoni, R., Gonzalez-Guzman, M., Rodriguez, L., Peirats-Llobet, M., Pizzio, G.A., Fernandez, M.A., De Winne, N., De Jaeger, G., Dietrich, D., Bennett, M.J*., et al.* (2013). PYRABACTIN RESISTANCE1-LIKE8 Plays an Important Role for the Regulation of Abscisic Acid Signaling in Root. Plant physiology *161*, 931-941.

Antoni, R., Gonzalez-Guzman, M., Rodriguez, L., Rodrigues, A., Pizzio, G.A., and Rodriguez, P.L. (2012). Selective inhibition of clade A phosphatases type 2C by PYR/PYL/RCAR abscisic acid receptors. Plant physiology *158*, 970-980.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. Nature *415*, 977-983.

Ascencio-Ibanez, J.T., Sozzani, R., Lee, T.J., Chu, T.M., Wolfinger, R.D., Cella, R., and Hanley-Bowdoin, L. (2008). Global analysis of Arabidopsis gene expression uncovers a complex array of changes impacting pathogen response and cell cycle during geminivirus infection. Plant physiology *148*, 436-454.

Assmann, S.M. (2003). OPEN STOMATA1 opens the door to ABA signaling in Arabidopsis guard cells. Trends in plant science *8*, 151-153.

Baena-Gonzalez, E., Rolland, F., Thevelein, J.M., and Sheen, J. (2007). A central integrator of transcription networks in plant stress and energy signalling. Nature *448*, 938-942.

Bai, C., and Elledge, S.J. (1996). Gene identification using the yeast two-hybrid system. Methods in Enzymology *273*, 331-347.

Baril, C., and Therrien, M. (2006). Alphabet, a Ser/Thr phosphatase of the protein phosphatase 2C family, negatively regulates RAS/MAPK signaling in Drosophila. Developmental biology *294*, 232-245.

Barrero, J.M., Piqueras, P., Gonzalez-Guzman, M., Serrano, R., Rodriguez, P.L., Ponce, M.R., and Micol, J.L. (2005). A mutational analysis of the ABA1 gene of Arabidopsis thaliana highlights the involvement of ABA in vegetative development. Journal of experimental botany *56*, 2071-2083.

Batistic, O., and Kudla, J. (2004). Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. Planta *219*, 915-924.

Batistic, O., and Kudla, J. (2009). Plant calcineurin B-like proteins and their interacting protein kinases. Biochimica et biophysica acta *1793*, 985-992.

Batistic, O., Waadt, R., Steinhorst, L., Held, K., and Kudla, J. (2010). CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. The Plant journal : for cell and molecular biology *61*, 211-222.

Ben-Ari, G. (2012). The ABA signal transduction mechanism in commercial crops: learning from Arabidopsis. Plant cell reports *31*, 1357-1369.

Benfey, P.N., and Chua, N.H. (1990). The Cauliflower Mosaic Virus 35S Promoter: Combinatorial Regulation of Transcription in Plants. Science *250*, 959-966.

Bertauche, N., Leung, J., and Giraudat, J. (1996). Protein phosphatase activity of abscisic acid insensitive 1 (ABI1) protein from Arabidopsis thaliana. European journal of biochemistry / FEBS *241*, 193-200.

Bhaskara, G.B., Nguyen, T.T., and Verslues, P.E. (2012). Unique Drought Resistance Functions of the Highly ABA-Induced Clade A Protein Phosphatase 2Cs. Plant physiology *160*, 379-395.

Bhattacharjee, S. (2005). Reactive oxygen species and oxidative burst: Roles in stress, senescence and signal transduction in plants. Curr Sci India *89*, 1113-1121.

Bhattacharya, A., Sood, P., and Citovsky, V. (2010). The roles of plant phenolics in defence and communication during Agrobacterium and Rhizobium infection. Molecular plant pathology *11*, 705-719.

Blanco, F., Salinas, P., Cecchini, N.M., Jordana, X., Van Hummelen, P., Alvarez, M.E., and Holuigue, L. (2009). Early genomic responses to salicylic acid in Arabidopsis. Plant molecular biology *70*, 79-102.

Boller, T., and He, S.Y. (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science *324*, 742-744.

Boudsocq, M., Barbier-Brygoo, H., and Lauriere, C. (2004). Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. The Journal of biological chemistry *279*, 41758-41766.

Boudsocq, M., and Sheen, J. (2012). CDPKs in immune and stress signaling. Trends in plant science.

Boudsocq, M., Willmann, M.R., McCormack, M., Lee, H., Shan, L., He, P., Bush, J., Cheng, S.H., and Sheen, J. (2010). Differential innate immune signalling via Ca(2+) sensor protein kinases. Nature *464*, 418-422.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., and Gorlach, J. (2001). Growth stage-based phenotypic analysis of Arabidopsis: a model for high throughput functional genomics in plants. The Plant cell *13*, 1499-1510.

Brandt, B., Brodsky, D.E., Xue, S., Negi, J., Iba, K., Kangasjarvi, J., Ghassemian, M., Stephan, A.B., Hu, H., and Schroeder, J.I. (2012). Reconstitution of abscisic acid activation of SLAC1 anion channel by CPK6 and OST1 kinases and branched ABI1 PP2C phosphatase action. Proceedings of the National Academy of Sciences of the United States of America *109*, 10593-10598.

Brock, A.K., Willmann, R., Kolb, D., Grefen, L., Lajunen, H.M., Bethke, G., Lee, J., Nurnberger, T., and Gust, A.A. (2010). The Arabidopsis mitogen-activated protein kinase phosphatase PP2C5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression. Plant physiology *153*, 1098-1111.

Brodersen, P., Petersen, M., Bjorn Nielsen, H., Zhu, S., Newman, M.A., Shokat, K.M., Rietz, S., Parker, J., and Mundy, J. (2006). Arabidopsis MAP kinase 4 regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4. The Plant journal : for cell and molecular biology *47*, 532-546.

Brody, M.S., Stewart, V., and Price, C.W. (2009). Bypass suppression analysis maps the signalling pathway within a multidomain protein: the RsbP energy stress phosphatase 2C from Bacillus subtilis. Molecular microbiology *72*, 1221-1234.

Busk, P.K., and Pages, M. (1998). Regulation of abscisic acid-induced transcription. Plant molecular biology *37*, 425-435.

Calderon Villalobos, L.I., Lee, S., De Oliveira, C., Ivetac, A., Brandt, W., Armitage, L., Sheard, L.B., Tan, X., Parry, G., Mao, H.*, et al.* (2012). A combinatorial TIR1/AFB-Aux/IAA co-receptor system for differential sensing of auxin. Nature chemical biology *8*, 477-485.

Cameron, R.K., Paiva, N.L., Lamb, C.J., and Dixon, R.A. (1999). Accumulation of salicylic acid and PR-1 gene transcripts in relation to the systemic acquired resistance (SAR) response induced by Pseudomonas syringae pv. tomato in Arabidopsis. Physiological and Molecular Plant Pathology *55*, 121-130.

Carrasco, J.L., Ancillo, G., Mayda, E., and Vera, P. (2003). A novel transcription factor involved in plant defense endowed with protein phosphatase activity. The EMBO journal *22*, 3376-3384.

Carrasco, J.L., Castello, M.J., and Vera, P. (2006). 14-3-3 mediates transcriptional regulation by modulating nucleocytoplasmic shuttling of tobacco DNA-binding protein phosphatase-1. The Journal of biological chemistry *281*, 22875-22881.

Castello, M.J., Carrasco, J.L., Navarrete-Gomez, M., Daniel, J., Granot, D., and Vera, P. (2011). A plant small polypeptide is a novel component of DNA-binding protein phosphatase 1-mediated resistance to plum pox virus in Arabidopsis. Plant physiology *157*, 2206-2215.

Castello, M.J., Carrasco, J.L., and Vera, P. (2010). DNA-binding protein phosphatase AtDBP1 mediates susceptibility to two potyviruses in Arabidopsis. Plant physiology *153*, 1521-1525.

Chakraborty, S., and Newton, A.C. (2011). Climate change, plant diseases and food security. Plant Pathol *60*, 2-14.

Chan, S.L., Low, L.Y., Hsu, S., Li, S., Liu, T., Santelli, E., Le Negrate, G., Reed, J.C., Woods, V.L., Jr., and Pascual, J. (2009). Molecular mimicry in innate immunity: crystal structure of a bacterial TIR domain. The Journal of biological chemistry *284*, 21386-21392.

Chan, Z. (2012). Expression profiling of ABA pathway transcripts indicates crosstalk between abiotic and biotic stress responses in Arabidopsis. Genomics *100*, 110-115.

Chang, K.F., Hwang, S.F., Ahmed, H.U., Strelkov, S.E., Conner, R.L., Gossen, B.D., Bing, D.J., and Turnbull, G.D. (2013). Yield loss and management of downy mildew on field pea in Alberta, Canada. Crop Protection *46*, 23-28.

Chatr-Aryamontri, A., Breitkreutz, B.J., Heinicke, S., Boucher, L., Winter, A., Stark, C., Nixon, J., Ramage, L., Kolas, N., O'Donnell, L.*, et al.* (2013). The BioGRID interaction database: 2013 update. Nucleic acids research *41*, D816-823.

Chaves, M.M., Flexas, J., and Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Annals of botany *103*, 551-560.

Chen, J.Y., and Dai, X.F. (2010). Cloning and characterization of the Gossypium hirsutum major latex protein gene and functional analysis in Arabidopsis thaliana. Planta *231*, 861-873.

Chen, L.C., Chen, J.C., Shu, J.C., Chen, C.Y., Chen, S.C., Chen, S.H., Lin, C.Y., Lu, C.Y., and Chen, C.C. (2012). Interplay of RsbM and RsbK controls the sigma(B) activity of Bacillus cereus. Environmental microbiology *14*, 2788-2799.

Chen, P.Y., Wang, C.K., Soong, S.C., and To, K.Y. (2003). Complete sequence of the binary vector pBI121 and its application in cloning T-DNA insertion from transgenic plants. Molecular Breeding *11*, 287-293.

Cheng, N.H., Pittman, J.K., Zhu, J.K., and Hirschi, K.D. (2004). The protein kinase SOS2 activates the Arabidopsis H(+)/Ca(2+) antiporter CAX1 to integrate calcium transport and salt tolerance. The Journal of biological chemistry *279*, 2922-2926.

Cheng, S.H., Willmann, M.R., Chen, H.C., and Sheen, J. (2002). Calcium signaling through protein kinases. The Arabidopsis calcium-dependent protein kinase gene family. Plant physiology *129*, 469-485.

Cheong, Y.H., Pandey, G.K., Grant, J.J., Batistic, O., Li, L., Kim, B.G., Lee, S.C., Kudla, J., and Luan, S. (2007). Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis. The Plant journal : for cell and molecular biology *52*, 223-239.

Cherel, I., Michard, E., Platet, N., Mouline, K., Alcon, C., Sentenac, H., and Thibaud, J.B. (2002). Physical and functional interaction of the Arabidopsis K(+) channel AKT2 and phosphatase AtPP2CA. The Plant cell *14*, 1133-1146.

Chibani, K., Ali-Rachedi, S., Job, C., Job, D., Jullien, M., and Grappin, P. (2006). Proteomic analysis of seed dormancy in Arabidopsis. Plant physiology *142*, 1493-1510.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. Nature *448*, 497-500.

Chinnusamy, V., Schumaker, K., and Zhu, J.K. (2004). Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. Journal of experimental botany *55*, 225-236.

Choi, H.I., Park, H.J., Park, J.H., Kim, S., Im, M.Y., Seo, H.H., Kim, Y.W., Hwang, I., and Kim, S.Y. (2005). Arabidopsis calcium-dependent protein kinase AtCPK32 interacts with ABF4, a transcriptional regulator of abscisic acid-responsive gene expression, and modulates its activity. Plant physiology *139*, 1750-1761.

Choudhury, A., and Lahiri, A. (2011). Comparative analysis of abscisic acid-regulated transcriptomes in Arabidopsis. Plant biology *13*, 28-35.

Christmann, A., Hoffmann, T., Teplova, I., Grill, E., and Muller, A. (2005). Generation of active pools of abscisic acid revealed by in vivo imaging of water-stressed Arabidopsis. Plant physiology *137*, 209-219.

Clark, B.J. (2012). The mammalian START domain protein family in lipid transport in health and disease. The Journal of endocrinology *212*, 257-275.

Coca, M., and San Segundo, B. (2010). AtCPK1 calcium-dependent protein kinase mediates pathogen resistance in Arabidopsis. The Plant journal : for cell and molecular biology.

Cohen, P. (1989). The structure and regulation of protein phosphatases. Annual review of biochemistry *58*, 453-508.

Consortium, A.I.M. (2011). Evidence for network evolution in an Arabidopsis interactome map. Science *333*, 601-607.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. Nucleic acids research *16*, 10881-10890.

Cuadrado, A., and Nebreda, A.R. (2010). Mechanisms and functions of p38 MAPK signalling. The Biochemical journal *429*, 403-417.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R., and Abrams, S.R. (2010). Abscisic acid: emergence of a core signaling network. Annual review of plant biology *61*, 651-679.

D'Angelo, C., Weinl, S., Batistic, O., Pandey, G.K., Cheong, Y.H., Schultke, S., Albrecht, V., Ehlert, B., Schulz, B., Harter, K.*, et al.* (2006). Alternative complex formation of the Ca-regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in Arabidopsis. The Plant journal : for cell and molecular biology *48*, 857-872.

Dahche, H., Abdullah, A., Ben Potters, M., and Kennelly, P.J. (2009). A PPM-family protein phosphatase from the thermoacidophile Thermoplasma volcanium hydrolyzes protein-bound phosphotyrosine. Extremophiles : life under extreme conditions *13*, 371-377.

Dangl, J.L., and Jones, J.D. (2001). Plant pathogens and integrated defence responses to infection. Nature *411*, 826-833.

Devos, S., Laukens, K., Deckers, P., Van Der Straeten, D., Beeckman, T., Inze, D., Van Onckelen, H., Witters, E., and Prinsen, E. (2006). A hormone and proteome approach to picturing the initial metabolic events during Plasmodiophora brassicae infection on Arabidopsis. Molecular plant-microbe interactions : MPMI *19*, 1431-1443.

Du, W., Lin, H., Chen, S., Wu, Y., Zhang, J., Fuglsang, A.T., Palmgren, M.G., Wu, W., and Guo, Y. (2011). Phosphorylation of SOS3-like calcium-binding proteins by their interacting SOS2-like protein kinases is a common regulatory mechanism in Arabidopsis. Plant physiology *156*, 2235-2243.

Dupeux, F., Santiago, J., Betz, K., Twycross, J., Park, S.Y., Rodriguez, L., Gonzalez-Guzman, M., Jensen, M.R., Krasnogor, N., Blackledge, M.*, et al.* (2011). A thermodynamic switch modulates abscisic acid receptor sensitivity. The EMBO journal *30*, 4171-4184.

Eichhorn, P.J., Creyghton, M.P., and Bernards, R. (2009). Protein phosphatase 2A regulatory subunits and cancer. Biochimica et biophysica acta *1795*, 1-15.

Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P., and Martin, G.B. (2003). Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. The Plant journal : for cell and molecular biology *36*, 905-917.

Farkas, I., Dombradi, V., Miskei, M., Szabados, L., and Koncz, C. (2007). Arabidopsis PPP family of serine/threonine phosphatases. Trends in plant science *12*, 169-176.

Felsenstein, J. (1985). Confidence-Limits on Phylogenies - an Approach Using the Bootstrap. Evolution *39*, 783-791.

Fernandes, H., Bujacz, A., Bujacz, G., Jelen, F., Jasinski, M., Kachlicki, P., Otlewski, J., Sikorski, M.M., and Jaskolski, M. (2009). Cytokinin-induced structural adaptability of a Lupinus luteus PR-10 protein. The FEBS journal *276*, 1596-1609.

Fernandes, H., Pasternak, O., Bujacz, G., Bujacz, A., Sikorski, M.M., and Jaskolski, M. (2008). Lupinus luteus pathogenesis-related protein as a reservoir for cytokinin. Journal of molecular biology *378*, 1040-1051.

Finkelstein, R.R., Gampala, S.S., and Rock, C.D. (2002). Abscisic acid signaling in seeds and seedlings. The Plant cell *14 Suppl*, S15-45.

Finkelstein, R.R., and Somerville, C.R. (1990). 3 Classes of Abscisic-Acid (Aba)-Insensitive Mutations of Arabidopsis Define Genes That Control Overlapping Subsets of Aba Responses. Plant physiology *94*, 1172-1179.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D*., et al.* (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature *422*, 442-446.

Franz, S., Ehlert, B., Liese, A., Kurth, J., Cazale, A.C., and Romeis, T. (2011). Calcium-dependent protein kinase CPK21 functions in abiotic stress response in Arabidopsis thaliana. Molecular plant *4*, 83-96.

Fu, Z.Q., and Dong, X. (2013). Systemic acquired resistance: turning local infection into global defense. Annual review of plant biology *64*, 839-863.

Fuchs, S., Grill, E., Meskiene, I., and Schweighofer, A. (2012). Type 2C protein phosphatases in plants. The FEBS journal.

Fuglsang, A.T., Guo, Y., Cuin, T.A., Qiu, Q., Song, C., Kristiansen, K.A., Bych, K., Schulz, A., Shabala, S., Schumaker, K.S*., et al.* (2007). Arabidopsis protein kinase PKS5 inhibits the plasma membrane H+ -ATPase by preventing interaction with 14-3-3 protein. The Plant cell *19*, 1617-1634.

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.Y., Cutler, S.R., Sheen, J., Rodriguez, P.L., and Zhu, J.K. (2009). In vitro reconstitution of an abscisic acid signalling pathway. Nature *462*, 660-664.

Fujii, H., Verslues, P.E., and Zhu, J.K. (2007). Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. The Plant cell *19*, 485-494.

Fujii, H., and Zhu, J.K. (2009). Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. Proceedings of the National Academy of Sciences of the United States of America *106*, 8380-8385.

Fujita, Y., Fujita, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2011). ABA-mediated transcriptional regulation in response to osmotic stress in plants. Journal of plant research *124*, 509-525.

Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N., Umezawa, T., Fujita, M., Maruyama, K., Ishiyama, K*., et al.* (2009). Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. Plant & cell physiology *50*, 2123-2132.

Fujita, Y., Yoshida, T., and Yamaguchi-Shinozaki, K. (2013). Pivotal role of the AREB/ABF-SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants. Physiologia plantarum *147*, 15-27.

Gaengel, K., and Mlodzik, M. (2003). Egfr signaling regulates ommatidial rotation and cell motility in the Drosophila eye via MAPK/Pnt signaling and the Ras effector Canoe/AF6. Development *130*, 5413-5423.

Gagne, J.M., and Clark, S.E. (2010). The Arabidopsis stem cell factor POLTERGEIST is membrane localized and phospholipid stimulated. The Plant cell *22*, 729-743.

Gajhede, M., Osmark, P., Poulsen, F.M., Ipsen, H., Larsen, J.N., vanNeerven, R.J.J., Schou, C., Lowenstein, H., and Spangfort, M.D. (1996). X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. Nat Struct Biol *3*, 1040-1045.

Gao, P., Kolenovsky, A., Cui, Y., Cutler, A.J., and Tsang, E.W. (2012). Expression, purification and analysis of an Arabidopsis recombinant CBL-interacting protein kinase3 (CIPK3) and its constitutively active form. Protein expression and purification *86*, 45-52.

Geiger, D., Maierhofer, T., Al-Rasheid, K.A., Scherzer, S., Mumm, P., Liese, A., Ache, P., Wellmann, C., Marten, I., Grill, E.*, et al.* (2011). Stomatal closure by fast abscisic acid signaling is mediated by the guard cell anion channel SLAH3 and the receptor RCAR1. Science signaling *4*, ra32.

Geiger, D., Scherzer, S., Mumm, P., Marten, I., Ache, P., Matschi, S., Liese, A., Wellmann, C., Al-Rasheid, K.A., Grill, E.*, et al.* (2010). Guard cell anion channel SLAC1 is regulated by CDPK protein kinases with distinct Ca2+ affinities. Proceedings of the National Academy of Sciences of the United States of America *107*, 8023-8028.

Geiger, D., Scherzer, S., Mumm, P., Stange, A., Marten, I., Bauer, H., Ache, P., Matschi, S., Liese, A., Al-Rasheid, K.A.*, et al.* (2009). Activity of guard cell anion channel SLAC1 is controlled by drought-stress signaling kinase-phosphatase pair. Proceedings of the National Academy of Sciences of the United States of America *106*, 21425-21430.

Gietz, R.D., TriggsRaine, B., Robbins, A., Graham, K.C., and Woods, R.A. (1997). Identification of proteins that interact with a protein of interest: Applications of the yeast two-hybrid system. Mol Cell Biochem *172*, 67-79.

Gietz, R.D., and Woods, R.A. (2002). Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods in Enzymology *350*, 87-96.

Golem, S., and Culver, J.N. (2003). Tobacco mosaic virus induced alterations in the gene expression profile of Arabidopsis thaliana. Molecular plant-microbe interactions : MPMI *16*, 681-688.

Goloudina, A.R., Tanoue, K., Hammann, A., Fourmaux, E., Le Guezennec, X., Bulavin, D.V., Mazur, S.J., Appella, E., Garrido, C., and Demidov, O.N. (2012). Wip1 promotes RUNX2-dependent apoptosis in p53-negative tumors and protects normal tissues during treatment with anticancer agents. Proceedings of the National Academy of Sciences of the United States of America *109*, E68-75.

Gong, D., Guo, Y., Schumaker, K.S., and Zhu, J.K. (2004). The SOS3 family of calcium sensors and SOS2 family of protein kinases in Arabidopsis. Plant physiology *134*, 919-926.

Gong, D., Zhang, C., Chen, X., Gong, Z., and Zhu, J.K. (2002). Constitutive activation and transgenic evaluation of the function of an arabidopsis PKS protein kinase. The Journal of biological chemistry *277*, 42088-42096.

Gonzalez-Guzman, M., Pizzio, G.A., Antoni, R., Vera-Sirera, F., Merilo, E., Bassel, G.W., Fernandez, M.A., Holdsworth, M.J., Perez-Amador, M.A., Kollist, H.*, et al.* (2012). Arabidopsis PYR/PYL/RCAR Receptors Play a Major Role in Quantitative Regulation of Stomatal Aperture and Transcriptional Response to Abscisic Acid. The Plant cell *24*, 2483-2496.

Gosti, F., Beaudoin, N., Serizet, C., Webb, A.A., Vartanian, N., and Giraudat, J. (1999). ABI1 protein phosphatase 2C is a negative regulator of abscisic acid signaling. The Plant cell *11*, 1897-1910.

Gosti, F., Bertauche, N., Vartanian, N., and Giraudat, J. (1995). Abscisic acid-dependent and -independent regulation of gene expression by progressive drought in Arabidopsis thaliana. Molecular & general genetics : MGG *246*, 10-18.

Guo, D., Wong, W.S., Xu, W.Z., Sun, F.F., Qing, D.J., and Li, N. (2011). Cis-cinnamic acid-enhanced 1 gene plays a role in regulation of Arabidopsis bolting. Plant molecular biology *75*, 481-495.

Guo, Y., Halfter, U., Ishitani, M., and Zhu, J.K. (2001). Molecular characterization of functional domains in the protein kinase SOS2 that is required for plant salt tolerance. The Plant cell *13*, 1383-1399.

Guo, Y., Qiu, Q.S., Quintero, F.J., Pardo, J.M., Ohta, M., Zhang, C., Schumaker, K.S., and Zhu, J.K. (2004). Transgenic evaluation of activated mutant alleles of SOS2 reveals a critical requirement for its kinase activity and C-terminal regulatory domain for salt tolerance in Arabidopsis thaliana. The Plant cell *16*, 435-449.

Guo, Y., Xiong, L., Song, C.P., Gong, D., Halfter, U., and Zhu, J.K. (2002). A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in Arabidopsis. Developmental cell *3*, 233-244.

Ha, B.H., Davis, M.J., Chen, C., Lou, H.J., Gao, J., Zhang, R., Krauthammer, M., Halaban, R., Schlessinger, J., Turk, B.E.*, et al.* (2012). Type II p21-activated kinases (PAKs) are regulated by an autoinhibitory pseudosubstrate. Proceedings of the National Academy of Sciences of the United States of America *109*, 16107-16112.

Halford, N.G., and Hardie, D.G. (1998). SNF1-related protein kinases: global regulators of carbon metabolism in plants? Plant molecular biology *37*, 735-748.

Halfter, U., Ishitani, M., and Zhu, J.K. (2000). The Arabidopsis SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proceedings of the National Academy of Sciences of the United States of America *97*, 3735-3740.

Hao, Q., Yin, P., Li, W., Wang, L., Yan, C., Lin, Z., Wu, J.Z., Wang, J., Yan, S.F., and Yan, N. (2011). The molecular basis of ABA-independent inhibition of PP2Cs by a subclass of PYL proteins. Molecular cell *42*, 662-672.

Hardie, D.G. (2007). AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nature reviews Molecular cell biology *8*, 774-785.

Harper, J.F., Huang, J.F., and Lloyd, S.J. (1994). Genetic identification of an autoinhibitor in CDPK, a protein kinase with a calmodulin-like domain. Biochemistry *33*, 7267-7277.

Harrison, S.J., Mott, E.K., Parsley, K., Aspinall, S., Gray, J.C., and Cottage, A. (2006). A rapid and robust method of identifying transformed Arabidopsis thaliana seedlings following floral dip transformation. Plant methods *2*, 19.

Hattori, T., Totsuka, M., Hobo, T., Kagaya, Y., and Yamamoto-Toyoda, A. (2002). Experimentally determined sequence requirement of ACGT-containing abscisic acid response element. Plant & cell physiology *43*, 136-140.

Held, K., Pascaud, F., Eckert, C., Gajdanowicz, P., Hashimoto, K., Corratge-Faillie, C., Offenborn, J.N., Lacombe, B., Dreyer, I., Thibaud, J.B.*, et al.* (2011). Calcium-dependent modulation and plasma membrane targeting of the AKT2 potassium channel by the CBL4/CIPK6 calcium sensor/protein kinase complex. Cell research *21*, 1116-1130.

Hermann, M.S. (2009). Regulation von NIMIN- und PR1-Genen aus Arabidopsis thaliana und Nicotiana tabacum in der Salicylat-abhängigen Pathogenabwehr.

Himmelbach, A., Hoffmann, T., Leube, M., Hohener, B., and Grill, E. (2002). Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis. The EMBO journal *21*, 3029-3038.

Hirayama, T., and Umezawa, T. (2010). The PP2C-SnRK2 complex: the central regulator of an abscisic acid signaling pathway. Plant signaling & behavior *5*, 160-163.

Hobo, T., Asada, M., Kowyama, Y., and Hattori, T. (1999). ACGT-containing abscisic acid response element (ABRE) and coupling element 3 (CE3) are functionally equivalent. The Plant journal : for cell and molecular biology *19*, 679-689.

Hoffmann, T. (2001). Signaltransduktion von Abscisinsäure in Arabidopsis thaliana: Transiente Expression in Protoplasten als Modellsystem. In Lehrstuhl für Botanik (München: Technische Universität München), pp. 159.

Hong, J.K., Lee, S.C., and Hwang, B.K. (2005). Activation of pepper basic PR-1 gene promoter during defense signaling to pathogen, abiotic and environmental stresses. Gene *356*, 169-180.

Hong, J.Y., Chae, M.J., Lee, I.S., Lee, Y.N., Nam, M.H., Kim, D.Y., Byun, M.O., and Yoon, I.S. (2011). Phosphorylation-mediated regulation of a rice ABA responsive element binding factor. Phytochemistry *72*, 27-36.

Hoth, S., Morgante, M., Sanchez, J.P., Hanafey, M.K., Tingey, S.V., and Chua, N.H. (2002). Genome-wide gene expression profiling in Arabidopsis thaliana reveals new targets of abscisic acid and largely impaired gene regulation in the abi1-1 mutant. Journal of cell science *115*, 4891-4900.

Hrabak, E.M., Chan, C.W., Gribskov, M., Harper, J.F., Choi, J.H., Halford, N., Kudla, J., Luan, S., Nimmo, H.G., Sussman, M.R.*, et al.* (2003). The Arabidopsis CDPK-SnRK superfamily of protein kinases. Plant physiology *132*, 666-680.

Hu, H.C., Wang, Y.Y., and Tsay, Y.F. (2009). AtCIPK8, a CBL-interacting protein kinase, regulates the low-affinity phase of the primary nitrate response. The Plant journal : for cell and molecular biology *57*, 264-278.

Hu, X., Liu, L., Xiao, B., Li, D., Xing, X., Kong, X., and Li, D. (2010). Enhanced tolerance to low temperature in tobacco by over-expression of a new maize protein phosphatase 2C, ZmPP2C2. Journal of plant physiology *167*, 1307-1315.

Huala, E., Dickerman, A.W., Garcia-Hernandez, M., Weems, D., Reiser, L., LaFond, F., Hanley, D., Kiphart, D., Zhuang, M., Huang, W.*, et al.* (2001). The Arabidopsis Information Resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. Nucleic acids research *29*, 102-105.

Huang, D., Jaradat, M.R., Wu, W., Ambrose, S.J., Ross, A.R., Abrams, S.R., and Cutler, A.J. (2007). Structural analogs of ABA reveal novel features of ABA perception and signaling in Arabidopsis. The Plant journal : for cell and molecular biology *50*, 414-428.

Huang, D., Wu, W., Abrams, S.R., and Cutler, A.J. (2008). The relationship of drought-related gene expression in Arabidopsis thaliana to hormonal and environmental factors. Journal of experimental botany *59*, 2991-3007.

Huang, J.F., Teyton, L., and Harper, J.F. (1996). Activation of a Ca(2+)-dependent protein kinase involves intramolecular binding of a calmodulin-like regulatory domain. Biochemistry *35*, 13222-13230.

Initiative, T.A.G. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature *408*, 796-815.

Ishida, A., Sueyoshi, N., Shigeri, Y., and Kameshita, I. (2008). Negative regulation of multifunctional Ca2+/calmodulin-dependent protein kinases: physiological and pharmacological significance of protein phosphatases. British journal of pharmacology *154*, 729-740.

Iyer, L.M., Koonin, E.V., and Aravind, L. (2001). Adaptations of the helix-grip fold for ligand binding and catalysis in the START domain superfamily. Proteins *43*, 134-144.

Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T., Parcy, F., and Group, b.I.P.R. (2002). bZIP transcription factors in Arabidopsis. Trends in plant science *7*, 106-111.

James, P., Halladay, J., and Craig, E.A. (1996). Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. Genetics *144*, 1425-1436.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep *5*, 387-405.

Jeong, H.J., Jwa, N.-S., and Kim, K.-N. (2005). Identification and characterization of protein kinases that interact with the CBL3 calcium sensor in Arabidopsis. Plant science : an international journal of experimental plant biology *169*, 1125-1135.

Jones, A.M., Thomas, V., Bennett, M.H., Mansfield, J., and Grant, M. (2006). Modifications to the Arabidopsis defense proteome occur prior to significant transcriptional change in response to inoculation with Pseudomonas syringae. Plant physiology *142*, 1603-1620.

Joshi-Saha, A., Valon, C., and Leung, J. (2011). Abscisic acid signal off the STARting block. Molecular plant *4*, 562-580.

Jossier, M., Bouly, J.P., Meimoun, P., Arjmand, A., Lessard, P., Hawley, S., Grahame Hardie, D., and Thomas, M. (2009). SnRK1 (SNF1-related kinase 1) has a central role in sugar and ABA signalling in Arabidopsis thaliana. The Plant journal : for cell and molecular biology *59*, 316-328.

Kanchiswamy, C.N., Takahashi, H., Quadro, S., Maffei, M.E., Bossi, S., Bertea, C., Zebelo, S.A., Muroi, A., Ishihama, N., Yoshioka, H.*, et al.* (2010). Regulation of Arabidopsis defense responses against Spodoptera littoralis by CPK-mediated calcium signaling. BMC plant biology *10*, 97.

Kang, J.Y., Choi, H.I., Im, M.Y., and Kim, S.Y. (2002). Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. The Plant cell *14*, 343-357.

Karssen, C.M., Brinkhorstvanderswan, D.L.C., Breekland, A.E., and Koornneef, M. (1983). Induction of Dormancy during Seed Development by Endogenous Abscisic-Acid - Studies on Abscisic-Acid Deficient Genotypes of Arabidopsis-Thaliana (L) Heynh. Planta *157*, 158-165.

Kerk, D., Templeton, G., and Moorhead, G.B. (2008). Evolutionary radiation pattern of novel protein phosphatases revealed by analysis of protein data from the completely sequenced genomes of humans, green algae, and higher plants. Plant physiology *146*, 351-367.

Kesarwani, M., Yoo, J., and Dong, X. (2007). Genetic interactions of TGA transcription factors in the regulation of pathogenesis-related genes and disease resistance in Arabidopsis. Plant physiology *144*, 336-346.

Kiegle, E., Moore, C.A., Haseloff, J., Tester, M.A., and Knight, M.R. (2000). Cell-type-specific calcium responses to drought, salt and cold in the Arabidopsis root. The Plant journal : for cell and molecular biology *23*, 267-278.

Kilian, J., Whitehead, D., Horak, J., Wanke, D., Weinl, S., Batistic, O., D'Angelo, C., Bornberg-Bauer, E., Kudla, J., and Harter, K. (2007). The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. The Plant journal : for cell and molecular biology *50*, 347-363.

Kim, K.N., Cheong, Y.H., Grant, J.J., Pandey, G.K., and Luan, S. (2003). CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in Arabidopsis. The Plant cell *15*, 411-423.

Kim, K.N., Cheong, Y.H., Gupta, R., and Luan, S. (2000). Interaction specificity of Arabidopsis calcineurin B-like calcium sensors and their target kinases. Plant physiology *124*, 1844-1853.

Kim, S., Kang, J.Y., Cho, D.I., Park, J.H., and Kim, S.Y. (2004a). ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. The Plant journal : for cell and molecular biology *40*, 75-87.

Kim, T.J., Gaidenko, T.A., and Price, C.W. (2004b). A multicomponent protein complex mediates environmental stress signaling in Bacillus subtilis. Journal of molecular biology *341*, 135-150.

Kimbrough, J.M., Salinas-Mondragon, R., Boss, W.F., Brown, C.S., and Sederoff, H.W. (2004). The fast and transient transcriptional network of gravity and mechanical stimulation in the Arabidopsis root apex. Plant physiology *136*, 2790-2805.

Klingler, J.P., Batelli, G., and Zhu, J.K. (2010). ABA receptors: the START of a new paradigm in phytohormone signalling. Journal of experimental botany *61*, 3199-3210.

Klusener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hugouvieux, V., and Schroeder, J.I. (2002). Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. Plant physiology *130*, 2152-2163.

Knight, H., Trewavas, A.J., and Knight, M.R. (1997). Calcium signalling in Arabidopsis thaliana responding to drought and salinity. The Plant journal : for cell and molecular biology *12*, 1067-1078.

Knutova, I. (2008). Functional characterization of the Ca2+-regulated kinase AtCIPK8 involved in ABA and oxidative stress responses.

Kobayashi, M., Ohura, I., Kawakita, K., Yokota, N., Fujiwara, M., Shimamoto, K., Doke, N., and Yoshioka, H. (2007). Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. The Plant cell *19*, 1065-1080.

Kobayashi, Y., Murata, M., Minami, H., Yamamoto, S., Kagaya, Y., Hobo, T., Yamamoto, A., and Hattori, T. (2005). Abscisic acid-activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. The Plant journal : for cell and molecular biology *44*, 939-949.

Kofler, S., Asam, C., Eckhard, U., Wallner, M., Ferreira, F., and Brandstetter, H. (2012). Crystallographically mapped ligand binding differs in high and low IgE binding isoforms of birch pollen allergen bet v 1. Journal of molecular biology *422*, 109-123.

Kolukisaoglu, U., Weinl, S., Blazevic, D., Batistic, O., and Kudla, J. (2004). Calcium sensors and their interacting protein kinases: genomics of the Arabidopsis and rice CBL-CIPK signaling networks. Plant physiology *134*, 43-58.

Koornneef, M., Reuling, G., and Karssen, C.M. (1984). The Isolation and Characterization of Abscisic-Acid Insensitive Mutants of Arabidopsis-Thaliana. Physiologia plantarum *61*, 377-383.

Kreps, J.A., Wu, Y., Chang, H.S., Zhu, T., Wang, X., and Harper, J.F. (2002). Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant physiology *130*, 2129-2141.

Krishnamurthy, L., Zaman-Allah, M., Purushothaman, R., Irshad, M., and Vadez, V. (2011). Plant Biomass Productivity Under Abiotic Stresses in SAT Agriculture. In Biomass - Detection, Production and Usage, D.D. Matovic, ed. (InTech), pp. 247-264.

Kudla, J., Xu, Q., Harter, K., Gruissem, W., and Luan, S. (1999). Genes for calcineurin B-like proteins in Arabidopsis are differentially regulated by stress signals. Proceedings of the National Academy of Sciences of the United States of America *96*, 4718-4723.

Kuhn, J.M., Boisson-Dernier, A., Dizon, M.B., Maktabi, M.H., and Schroeder, J.I. (2006). The protein phosphatase AtPP2CA negatively regulates abscisic acid signal transduction in Arabidopsis, and effects of abh1 on AtPP2CA mRNA. Plant physiology *140*, 127-139.

Kulik, A., Wawer, I., Krzywinska, E., Bucholc, M., and Dobrowolska, G. (2011). SnRK2 protein kinases--key regulators of plant response to abiotic stresses. Omics : a journal of integrative biology *15*, 859-872.

Kurien, B.T., and Scofield, R.H. (1998). Heat mediated quick Coomassie blue protein staining and destaining of SDS-PAGE gels. Indian journal of biochemistry & biophysics *35*, 385-389.

Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D., and Schroeder, J.I. (2003). NADPH oxidase AtrbohD and AtrbohF genes function in ROS-dependent ABA signaling in Arabidopsis. The EMBO journal *22*, 2623-2633.

Kwak, J.M., Nguyen, V., and Schroeder, J.I. (2006). The role of reactive oxygen species in hormonal responses. Plant physiology *141*, 323-329.

Lackman, P., Gonzalez-Guzman, M., Tilleman, S., Carqueijeiro, I., Perez, A.C., Moses, T., Seo, M., Kanno, Y., Hakkinen, S.T., Van Montagu, M.C*., et al.* (2011). Jasmonate signaling involves the abscisic acid receptor PYL4 to regulate metabolic reprogramming in Arabidopsis and tobacco. Proceedings of the National Academy of Sciences of the United States of America *108*, 5891-5896.

Lamb, C., and Dixon, R.A. (1997). The Oxidative Burst in Plant Disease Resistance. Annual review of plant physiology and plant molecular biology *48*, 251-275.

Lan, W.Z., Lee, S.C., Che, Y.F., Jiang, Y.Q., and Luan, S. (2011). Mechanistic analysis of AKT1 regulation by the CBL-CIPK-PP2CA interactions. Molecular plant *4*, 527-536.

Larcher, W. (1987). Streß bei Pflanzen. Naturwissenschaften *74*, 158-167.

Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E. (1998). Functional analysis of regulatory sequences controlling PR-1 gene expression in Arabidopsis. The Plant journal : for cell and molecular biology *16*, 223-233.

Lecourieux, D., Ranjeva, R., and Pugin, A. (2006). Calcium in plant defence-signalling pathways. The New phytologist *171*, 249-269.

Lee, E.J., Iai, H., Sano, H., and Koizumi, N. (2005). Sugar responsible and tissue specific expression of a gene encoding AtCIPK14, an Arabidopsis CBL-interacting protein kinase. Bioscience, Biotechnology, and Biochemistry *69*, 242-245.

Lee, M.W., Jelenska, J., and Greenberg, J.T. (2008). Arabidopsis proteins important for modulating defense responses to Pseudomonas syringae that secrete HopW1-1. The Plant journal : for cell and molecular biology *54*, 452-465.

Lee, S.C., Lan, W., Buchanan, B.B., and Luan, S. (2009). A protein kinase-phosphatase pair interacts with an ion channel to regulate ABA signaling in plant guard cells. Proceedings of the National Academy of Sciences of the United States of America *106*, 21419-21424.

Lee, S.C., Lan, W.Z., Kim, B.G., Li, L., Cheong, Y.H., Pandey, G.K., Lu, G., Buchanan, B.B., and Luan, S. (2007). A protein phosphorylation/dephosphorylation network regulates a plant potassium channel. Proceedings of the National Academy of Sciences of the United States of America *104*, 15959-15964.

Lee, S.C., Lim, C.W., Lan, W., He, K., and Luan, S. (2012). ABA Signaling in Guard Cells Entails a Dynamic Protein-Protein Interaction Relay from the PYL-RCAR Family Receptors to Ion Channels. Molecular plant.

Leonhardt, N., Kwak, J.M., Robert, N., Waner, D., Leonhardt, G., and Schroeder, J.I. (2004). Microarray expression analyses of Arabidopsis guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. The Plant cell *16*, 596-615.

Leube, M.P., Grill, E., and Amrhein, N. (1998). ABI1 of Arabidopsis is a protein serine/threonine phosphatase highly regulated by the proton and magnesium ion concentration. FEBS letters *424*, 100-104.

Leung, J., Bouvier-Durand, M., Morris, P.C., Guerrier, D., Chefdor, F., and Giraudat, J. (1994). Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. Science *264*, 1448-1452.

Leung, J., Merlot, S., and Giraudat, J. (1997). The Arabidopsis ABSCISIC ACID-INSENSITIVE2 (ABI2) and ABI1 genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. The Plant cell *9*, 759-771.

Levin, E.J., Kondrashov, D.A., Wesenberg, G.E., and Phillips, G.N., Jr. (2007). Ensemble refinement of protein crystal structures: validation and application. Structure *15*, 1040-1052.

Li, L., Kim, B.G., Cheong, Y.H., Pandey, G.K., and Luan, S. (2006). A Ca(2)+ signaling pathway regulates a K(+) channel for low-K response in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America *103*, 12625-12630.

Lichtenthaler, H.K. (1996). Vegetation Stress: an Introduction to the Stress Concept in Plants. Journal of plant physiology *148*, 4-14.

Lin, P.C., Hwang, S.G., Endo, A., Okamoto, M., Koshiba, T., and Cheng, W.H. (2007). Ectopic expression of ABSCISIC ACID 2/GLUCOSE INSENSITIVE 1 in Arabidopsis promotes seed dormancy and stress tolerance. Plant physiology *143*, 745-758.

Little, D.P., Moran, R.C., Brenner, E.D., and Stevenson, D.W. (2007). Nuclear genome size in Selaginella. Genome / National Research Council Canada = Genome / Conseil national de recherches Canada *50*, 351-356.

Liu, H., and Naismith, J.H. (2008). An efficient one-step site-directed deletion, insertion, single and multiple-site plasmid mutagenesis protocol. BMC biotechnology *8*, 91.

Liu, J.-J., and Ekramoddoullah, A.K.M. (2006). The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. Physiological and Molecular Plant Pathology *68*, 3-13.

Liu, J., and Zhu, J.K. (1998). A calcium sensor homolog required for plant salt tolerance. Science *280*, 1943-1945.

Liu, L., Hu, X., Song, J., Zong, X., Li, D., and Li, D. (2009). Over-expression of a Zea mays L. protein phosphatase 2C gene (ZmPP2C) in Arabidopsis thaliana decreases tolerance to salt and drought. Journal of plant physiology *166*, 531-542.

Lobell, D.B., Cassman, K.G., and Field, C.B. (2009). Crop Yield Gaps: Their Importance, Magnitudes, and Causes. Annual Review of Environment and Resources *34*, 179-204.

Lopez-Molina, L., Mongrand, S., McLachlin, D.T., Chait, B.T., and Chua, N.H. (2002). ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. The Plant journal : for cell and molecular biology *32*, 317-328.

Lu, X., Nannenga, B., and Donehower, L.A. (2005). PPM1D dephosphorylates Chk1 and p53 and abrogates cell cycle checkpoints. Genes & development *19*, 1162-1174.

Luan, S., Lan, W., and Chul Lee, S. (2009). Potassium nutrition, sodium toxicity, and calcium signaling: connections through the CBL-CIPK network. Current opinion in plant biology *12*, 339-346.

Ludwig, A.A., Saitoh, H., Felix, G., Freymark, G., Miersch, O., Wasternack, C., Boller, T., Jones, J.D., and Romeis, T. (2005). Ethylene-mediated cross-talk between calcium-dependent protein kinase and MAPK signaling controls stress responses in plants. Proceedings of the National Academy of Sciences of the United States of America *102*, 10736-10741.

Lynch, T., Erickson, B.J., and Finkelstein, R.R. (2012). Direct interactions of ABA-insensitive(ABI)-clade protein phosphatase(PP)2Cs with calcium-dependent protein kinases and ABA response element-binding bZIPs may contribute to turning off ABA response. Plant molecular biology.

Lytle, B.L., Song, J., de la Cruz, N.B., Peterson, F.C., Johnson, K.A., Bingman, C.A., Phillips, G.N., Jr., and Volkman, B.F. (2009). Structures of two Arabidopsis thaliana major latex proteins represent novel helix-grip folds. Proteins *76*, 237-243.

Ma, S.Y., and Wu, W.H. (2007). AtCPK23 functions in Arabidopsis responses to drought and salt stresses. Plant molecular biology *65*, 511-518.

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christmann, A., and Grill, E. (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. Science *324*, 1064-1068.

Mahalingam, R., Gomez-Buitrago, A., Eckardt, N., Shah, N., Guevara-Garcia, A., Day, P., Raina, R., and Fedoroff, N.V. (2003). Characterizing the stress/defense transcriptome of Arabidopsis. Genome biology *4*, R20.

Mapes, J., and Ota, I.M. (2004). Nbp2 targets the Ptc1-type 2C Ser/Thr phosphatase to the HOG MAPK pathway. The EMBO journal *23*, 302-311.

Marković-Housley, Z., Degano, M., Lamba, D., von Roepenack-Lahaye, E., Clemens, S., Susani, M., Ferreira, F., Scheiner, O., and Breiteneder, H. (2003). Crystal Structure of a Hypoallergenic Isoform of the Major Birch Pollen Allergen Bet v 1 and its Likely Biological Function as a Plant Steroid Carrier. Journal of molecular biology *325*, 123-133.

Meinhard, M., and Grill, E. (2001). Hydrogen peroxide is a regulator of ABI1, a protein phosphatase 2C from Arabidopsis. FEBS letters *508*, 443-446.

Meinhard, M., Rodriguez, P.L., and Grill, E. (2002). The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signalling. Planta *214*, 775-782.

Melcher, K., Ng, L.M., Zhou, X.E., Soon, F.F., Xu, Y., Suino-Powell, K.M., Park, S.Y., Weiner, J.J., Fujii, H., Chinnusamy, V.*, et al.* (2009). A gate-latch-lock mechanism for hormone signalling by abscisic acid receptors. Nature *462*, 602-608.

Melcher, K., Xu, Y., Ng, L.M., Zhou, X.E., Soon, F.F., Chinnusamy, V., Suino-Powell, K.M., Kovach, A., Tham, F.S., Cutler, S.R.*, et al.* (2010a). Identification and mechanism of ABA receptor antagonism. Nature structural & molecular biology *17*, 1102-1108.

Melcher, K., Zhou, X.E., and Xu, H.E. (2010b). Thirsty plants and beyond: structural mechanisms of abscisic acid perception and signaling. Current opinion in structural biology *20*, 722-729.

Merlot, S., Gosti, F., Guerrier, D., Vavasseur, A., and Giraudat, J. (2001). The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signalling pathway. The Plant journal : for cell and molecular biology *25*, 295-303.

Meskiene, I., Baudouin, E., Schweighofer, A., Liwosz, A., Jonak, C., Rodriguez, P.L., Jelinek, H., and Hirt, H. (2003). Stress-induced protein phosphatase 2C is a negative regulator of a mitogen-activated protein kinase. The Journal of biological chemistry *278*, 18945-18952.

Meskiene, I., Bogre, L., Glaser, W., Balog, J., Brandstotter, M., Zwerger, K., Ammerer, G., and Hirt, H. (1998). MP2C, a plant protein phosphatase 2C, functions as a negative regulator of mitogen-activated protein kinase pathways in yeast and plants. Proceedings of the National Academy of Sciences of the United States of America *95*, 1938-1943.

Meyer, K., Leube, M.P., and Grill, E. (1994). A protein phosphatase 2C involved in ABA signal transduction in Arabidopsis thaliana. Science *264*, 1452-1455.

Miao, Y., Lv, D., Wang, P., Wang, X.C., Chen, J., Miao, C., and Song, C.P. (2006). An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. The Plant cell *18*, 2749-2766.

Mihaylova, M.M., and Shaw, R.J. (2011). The AMPK signalling pathway coordinates cell growth, autophagy and metabolism. Nature cell biology *13*, 1016-1023.

Miyakawa, T., Fujita, Y., Yamaguchi-Shinozaki, K., and Tanokura, M. (2012). Structure and function of abscisic acid receptors. Trends in plant science.

Miyazono, K., Miyakawa, T., Sawano, Y., Kubota, K., Kang, H.J., Asano, A., Miyauchi, Y., Takahashi, M., Zhi, Y., Fujita, Y.*, et al.* (2009). Structural basis of abscisic acid signalling. Nature *462*, 609-614.

Moes, D., Himmelbach, A., Korte, A., Haberer, G., and Grill, E. (2008). Nuclear localization of the mutant protein phosphatase abi1 is required for insensitivity towards ABA responses in Arabidopsis. The Plant journal : for cell and molecular biology *54*, 806-819.

Mogensen, J.E., Wimmer, R., Larsen, J.N., Spangfort, M.D., and Otzen, D.E. (2002). The major birch allergen, Bet v 1, shows affinity for a broad spectrum of physiological ligands. The Journal of biological chemistry *277*, 23684-23692.

Moorhead, G.B., De Wever, V., Templeton, G., and Kerk, D. (2009). Evolution of protein phosphatases in plants and animals. The Biochemical journal *417*, 401-409.

Moorhead, G.B., Trinkle-Mulcahy, L., and Ulke-Lemee, A. (2007). Emerging roles of nuclear protein phosphatases. Nature reviews Molecular cell biology *8*, 234-244.

Mori, I.C., and Schroeder, J.I. (2004). Reactive oxygen species activation of plant Ca2+ channels. A signaling mechanism in polar growth, hormone transduction, stress signaling, and hypothetically mechanotransduction. Plant physiology *135*, 702-708.

Mosquna, A., Peterson, F.C., Park, S.Y., Lozano-Juste, J., Volkman, B.F., and Cutler, S.R. (2011). Potent and selective activation of abscisic acid receptors in vivo by mutational stabilization of their agonist-bound conformation. Proceedings of the National Academy of Sciences of the United States of America *108*, 20838-20843.

Msanne, J., Lin, J., Stone, J.M., and Awada, T. (2011). Characterization of abiotic stress-responsive Arabidopsis thaliana RD29A and RD29B genes and evaluation of transgenes. Planta *234*, 97-107.

Munemasa, S., Hossain, M.A., Nakamura, Y., Mori, I.C., and Murata, Y. (2011). The Arabidopsis calcium-dependent protein kinase, CPK6, functions as a positive regulator of methyl jasmonate signaling in guard cells. Plant physiology *155*, 553-561.

Muniz Garcia, M.N., Giammaria, V., Grandellis, C., Tellez-Inon, M.T., Ulloa, R.M., and Capiati, D.A. (2012). Characterization of StABF1, a stress-responsive bZIP transcription factor from Solanum tuberosum L. that is phosphorylated by StCDPK2 in vitro. Planta *235*, 761-778.

Murashige, T., and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia plantarum *15*, 473-497.

Murray, D.A.H., Clarke, M.B., and Ronning, D.A. (2013). Estimating invertebrate pest losses in six major Australian grain crops. Australian Journal of Entomology, n/a-n/a.

Nakashima, K., Fujita, Y., Kanamori, N., Katagiri, T., Umezawa, T., Kidokoro, S., Maruyama, K., Yoshida, T., Ishiyama, K., Kobayashi, M.*, et al.* (2009). Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2,

SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. Plant & cell physiology *50*, 1345-1363.

Nakashima, K., Fujita, Y., Katsura, K., Maruyama, K., Narusaka, Y., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2006). Transcriptional regulation of ABI3- and ABA-responsive genes including RD29B and RD29A in seeds, germinating embryos, and seedlings of Arabidopsis. Plant molecular biology *60*, 51-68.

Navarova, H., Bernsdorff, F., Doring, A.C., and Zeier, J. (2012). Pipecolic acid, an endogenous mediator of defense amplification and priming, is a critical regulator of inducible plant immunity. The Plant cell *24*, 5123-5141.

Nessler, C.L., Kurz, W.G., and Pelcher, L.E. (1990). Isolation and analysis of the major latex protein genes of opium poppy. Plant molecular biology *15*, 951-953.

Ng, L.M., Soon, F.F., Zhou, X.E., West, G.M., Kovach, A., Suino-Powell, K.M., Chalmers, M.J., Li, J., Yong, E.L., Zhu, J.K.*, et al.* (2011). Structural basis for basal activity and autoactivation of abscisic acid (ABA) signaling SnRK2 kinases. Proceedings of the National Academy of Sciences of the United States of America *108*, 21259-21264.

Nishimura, N., Hitomi, K., Arvai, A.S., Rambo, R.P., Hitomi, C., Cutler, S.R., Schroeder, J.I., and Getzoff, E.D. (2009). Structural mechanism of abscisic acid binding and signaling by dimeric PYR1. Science *326*, 1373-1379.

Nishimura, N., Sarkeshik, A., Nito, K., Park, S.Y., Wang, A., Carvalho, P.C., Lee, S., Caddell, D.F., Cutler, S.R., Chory, J.*, et al.* (2010). PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. The Plant journal : for cell and molecular biology *61*, 290-299.

Nishimura, N., Yoshida, T., Kitahata, N., Asami, T., Shinozaki, K., and Hirayama, T. (2007). ABA-Hypersensitive Germination1 encodes a protein phosphatase 2C, an essential component of abscisic acid signaling in Arabidopsis seed. The Plant journal : for cell and molecular biology *50*, 935-949.

Nishimura, N., Yoshida, T., Murayama, M., Asami, T., Shinozaki, K., and Hirayama, T. (2004). Isolation and characterization of novel mutants affecting the abscisic acid sensitivity of Arabidopsis germination and seedling growth. Plant & cell physiology *45*, 1485-1499.

Nozawa, A., Sawada, Y., Akiyama, T., Koizumi, N., and Sano, H. (2003). Variable interactions between sucrose non-fermented 1-related protein kinases and regulatory proteins in higher plants. Bioscience, Biotechnology, and Biochemistry *67*, 2533-2540.

O'Donnell, P.J., Schmelz, E., Block, A., Miersch, O., Wasternack, C., Jones, J.B., and Klee, H.J. (2003). Multiple hormones act sequentially to mediate a susceptible tomato pathogen defense response. Plant physiology *133*, 1181-1189.

Odell, J.T., Nagy, F., and Chua, N.H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature *313*, 810-812.

Ohkuma, K., Lyon, J.L., Addicott, F.T., and Smith, O.E. (1963). Abscisin II, an Abscission-Accelerating Substance from Young Cotton Fruit. Science *142*, 1592-1593.

Ohta, M., Guo, Y., Halfter, U., and Zhu, J.K. (2003). A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. Proceedings of the National Academy of Sciences of the United States of America *100*, 11771-11776.

Onouchi, T., Sueyoshi, N., Ishida, A., and Kameshita, I. (2012). Phosphorylation and activation of nuclear Ca2+/calmodulin-dependent protein kinase phosphatase (CaMKP-N/PPM1E) by Ca2+/calmodulin-dependent protein kinase I (CaMKI). Biochemical and biophysical research communications *422*, 703-709.

Osmark, P., Boyle, B., and Brisson, N. (1998). Sequential and structural homology between intracellular pathogenesis-related proteins and a group of latex proteins. Plant molecular biology *38*, 1243-1246.

Overmyer, K., Brosché, M., and Kangasjärvi, J. (2003). Reactive oxygen species and hormonal control of cell death. Trends in plant science *8*, 335-342.

Pandey, G.K. (2008). Emergence of a novel calcium signaling pathway in plants: CBL-CIPK signaling network. Physiology and Molecular Biology of Plants *14*, 51-68.

Pandey, G.K., Cheong, Y.H., Kim, B.G., Grant, J.J., Li, L., and Luan, S. (2007). CIPK9: a calcium sensor-interacting protein kinase required for low-potassium tolerance in Arabidopsis. Cell research *17*, 411-421.

Pandey, G.K., Grant, J.J., Cheong, Y.H., Kim, B.G., Li le, G., and Luan, S. (2008). Calcineurin-B-like protein CBL9 interacts with target kinase CIPK3 in the regulation of ABA response in seed germination. Molecular plant *1*, 238-248.

Pape, S., Thurow, C., and Gatz, C. (2010). The Arabidopsis PR-1 promoter contains multiple integration sites for the coactivator NPR1 and the repressor SNI1. Plant physiology *154*, 1805-1818.

Park, S.Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T.F.*, et al.* (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. Science *324*, 1068-1071.

Pasternak, O., Bujacz, G.D., Fujimoto, Y., Hashimoto, Y., Jelen, F., Otlewski, J., Sikorski, M.M., and Jaskolski, M. (2006). Crystal structure of Vigna radiata cytokinin-specific binding protein in complex with zeatin. The Plant cell *18*, 2622-2634.

Peterson, F.C., Burgie, E.S., Park, S.Y., Jensen, D.R., Weiner, J.J., Bingman, C.A., Chang, C.E., Cutler, S.R., Phillips, G.N., Jr., and Volkman, B.F. (2010). Structural basis for selective activation of ABA receptors. Nature structural & molecular biology *17*, 1109-1113.

Phee, B.K., Kim, J.I., Shin, D.H., Yoo, J., Park, K.J., Han, Y.J., Kwon, Y.K., Cho, M.H., Jeon, J.S., Bhoo, S.H.*, et al.* (2008). A novel protein phosphatase indirectly regulates phytochrome-interacting factor 3 via phytochrome. The Biochemical journal *415*, 247-255.

Piao, H.L., Xuan, Y.H., Park, S.H., Je, B.I., Park, S.J., Park, S.H., Kim, C.M., Huang, J., Wang, G.K., Kim, M.J.*, et al.* (2010). OsCIPK31, a CBL-interacting protein kinase is involved in germination and seedling growth under abiotic stress conditions in rice plants. Molecules and cells *30*, 19-27.

Pirovano, W., Feenstra, K.A., and Heringa, J. (2008). PRALINETM: a strategy for improved multiple alignment of transmembrane proteins. Bioinformatics *24*, 492-497.

Pourtau, N., Mares, M., Purdy, S., Quentin, N., Ruel, A., and Wingler, A. (2004). Interactions of abscisic acid and sugar signalling in the regulation of leaf senescence. Planta *219*, 765-772.

Price, C.T., Al-Khodor, S., Al-Quadan, T., Santic, M., Habyarimana, F., Kalia, A., and Kwaik, Y.A. (2009). Molecular mimicry by an F-box effector of Legionella pneumophila hijacks a conserved polyubiquitination machinery within macrophages and protozoa. PLoS pathogens *5*, e1000704.

Qiu, J.L., Zhou, L., Yun, B.W., Nielsen, H.B., Fiil, B.K., Petersen, K., Mackinlay, J., Loake, G.J., Mundy, J., and Morris, P.C. (2008). Arabidopsis mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. Plant physiology *148*, 212-222.

Radauer, C., Lackner, P., and Breiteneder, H. (2008). The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. BMC evolutionary biology *8*, 286.

Radchuk, R., Emery, R.J., Weier, D., Vigeolas, H., Geigenberger, P., Lunn, J.E., Feil, R., Weschke, W., and Weber, H. (2010). Sucrose non-fermenting kinase 1 (SnRK1) coordinates metabolic and hormonal signals during pea cotyledon growth and differentiation. The Plant journal : for cell and molecular biology *61*, 324-338.

Raghavendra, A.S., Gonugunta, V.K., Christmann, A., and Grill, E. (2010). ABA perception and signalling. Trends in plant science *15*, 395-401.

Ramagli, L.S., and Rodriguez, L.V. (1985). Quantitation of Microgram Amounts of Protein in Two-Dimensional Polyacrylamide-Gel Electrophoresis Sample Buffer. Electrophoresis *6*, 559-563.

Razem, F.A., Baron, K., and Hill, R.D. (2006). Turning on gibberellin and abscisic acid signaling. Current opinion in plant biology *9*, 454-459.

Remmert, M., Biegert, A., Hauser, A., and Soding, J. (2012). HHblits: lightning-fast iterative protein sequence searching by HMM-HMM alignment. Nature methods *9*, 173-175.

Ren, X.L., Qi, G.N., Feng, H.Q., Zhao, S., Zhao, S.S., Wang, Y., and Wu, W.H. (2013). Calcineurin B-like protein CBL10 directly interacts with AKT1 and modulates K(+) homeostasis in Arabidopsis. The Plant journal : for cell and molecular biology.

Rensing, S.A., Lang, D., Zimmer, A.D., Terry, A., Salamov, A., Shapiro, H., Nishiyama, T., Perroud, P.F., Lindquist, E.A., Kamisugi, Y.*, et al.* (2008). The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. Science *319*, 64-69.

Robert, N., Merlot, S., N'Guyen, V., Boisson-Dernier, A., and Schroeder, J.I. (2006). A hypermorphic mutation in the protein phosphatase 2C HAB1 strongly affects ABA signaling in Arabidopsis. FEBS letters *580*, 4691-4696.

Rock, C.D., and Zeevaart, J.A. (1991). The aba mutant of Arabidopsis thaliana is impaired in epoxy-carotenoid biosynthesis. Proceedings of the National Academy of Sciences of the United States of America *88*, 7496-7499.

Rodriguez, M.C., Petersen, M., and Mundy, J. (2010). Mitogen-activated protein kinase signaling in plants. Annual review of plant biology *61*, 621-649.

Rodriguez, P.L., Benning, G., and Grill, E. (1998a). ABI2, a second protein phosphatase 2C involved in abscisic acid signal transduction in Arabidopsis. FEBS letters *421*, 185-190.

Rodriguez, P.L., Leube, M.P., and Grill, E. (1998b). Molecular cloning in Arabidopsis thaliana of a new protein phosphatase 2C (PP2C) with homology to ABI1 and ABI2. Plant molecular biology *38*, 879-883.

Roy, J., and Cyert, M.S. (2009). Cracking the phosphatase code: docking interactions determine substrate specificity. Science signaling *2*, re9.

Rubio, S., Rodrigues, A., Saez, A., Dizon, M.B., Galle, A., Kim, T.H., Santiago, J., Flexas, J., Schroeder, J.I., and Rodriguez, P.L. (2009). Triple loss of function of protein phosphatases type 2C leads to partial constitutive response to endogenous abscisic acid. Plant physiology *150*, 1345-1355.

Ruperti, B., Bonghi, C., Ziliotto, F., Pagni, S., Rasori, A., Varotto, S., Tonutti, P., Giovannoni, J.J., and Ramina, A. (2002). Characterization of a major latex protein (MLP) gene down-regulated by ethylene during peach fruitlet abscission. Plant science : an international journal of experimental plant biology *163*, 265-272.

Rushton, D.L., Tripathi, P., Rabara, R.C., Lin, J., Ringler, P., Boken, A.K., Langum, T.J., Smidt, L., Boomsma, D.D., Emme, N.J*., et al.* (2012). WRKY transcription factors: key components in abscisic acid signalling. Plant biotechnology journal *10*, 2-11.

Sacco, F., Perfetto, L., Castagnoli, L., and Cesareni, G. (2012). The human phosphatase interactome: An intricate family portrait. FEBS letters *586*, 2732-2739.

Saez, A., Apostolova, N., Gonzalez-Guzman, M., Gonzalez-Garcia, M.P., Nicolas, C., Lorenzo, O., and Rodriguez, P.L. (2004). Gain-of-function and loss-of-function phenotypes of the protein phosphatase 2C HAB1 reveal its role as a negative regulator of abscisic acid signalling. Plant Journal *37*, 354-369.

Saez, A., Robert, N., Maktabi, M.H., Schroeder, J.I., Serrano, R., and Rodriguez, P.L. (2006). Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. Plant physiology *141*, 1389-1399.

Sambrook, J., and Russell, D.W. (2001). Molecular Cloning, Third Edition edn (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sanchez-Barrena, M.J., Martinez-Ripoll, M., and Albert, A. (2013). Structural Biology of a Major Signaling Network that Regulates Plant Abiotic Stress: The CBL-CIPK Mediated Pathway. International journal of molecular sciences *14*, 5734-5749.

Santiago, J., Dupeux, F., Betz, K., Antoni, R., Gonzalez-Guzman, M., Rodriguez, L., Marquez, J.A., and Rodriguez, P.L. (2012). Structural insights into PYR/PYL/RCAR ABA receptors and PP2Cs. Plant science : an international journal of experimental plant biology *182*, 3-11.

Santiago, J., Dupeux, F., Round, A., Antoni, R., Park, S.Y., Jamin, M., Cutler, S.R., Rodriguez, P.L., and Marquez, J.A. (2009a). The abscisic acid receptor PYR1 in complex with abscisic acid. Nature *462*, 665-668.

Santiago, J., Rodrigues, A., Saez, A., Rubio, S., Antoni, R., Dupeux, F., Park, S.Y., Marquez, J.A., Cutler, S.R., and Rodriguez, P.L. (2009b). Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. The Plant journal : for cell and molecular biology *60*, 575-588.

Sato, A., Sato, Y., Fukao, Y., Fujiwara, M., Umezawa, T., Shinozaki, K., Hibi, T., Taniguchi, M., Miyake, H., Goto, D.B*., et al.* (2009). Threonine at position 306 of the KAT1 potassium channel is essential for channel activity and is a target site for ABA-activated SnRK2/OST1/SnRK2.6 protein kinase. The Biochemical journal *424*, 439-448.

Schena, M., and Davis, R.W. (1992). HD-Zip proteins: members of an Arabidopsis homeodomain protein superfamily. Proceedings of the National Academy of Sciences of the United States of America *89*, 3894-3898.

Scherzer, S., Maierhofer, T., Al-Rasheid, K.A., Geiger, D., and Hedrich, R. (2012). Multiple calcium-dependent kinases modulate ABA-activated guard cell anion channels. Molecular plant *5*, 1409-1412.

Schmid, M., Davison, T.S., Henz, S.R., Pape, U.J., Demar, M., Vingron, M., Scholkopf, B., Weigel, D., and Lohmann, J.U. (2005). A gene expression map of Arabidopsis thaliana development. Nature genetics *37*, 501-506.

Schmidt, C., Schelle, I., Liao, Y.J., and Schroeder, J.I. (1995). Strong regulation of slow anion channels and abscisic acid signaling in guard cells by phosphorylation and dephosphorylation events. Proceedings of the National Academy of Sciences of the United States of America *92*, 9535-9539.

Schrodinger, L. (2010). The PyMOL Molecular Graphics System, Version 1.3r1 (New York).

Schroeder, J.I., Kwak, J.M., and Allen, G.J. (2001). Guard cell abscisic acid signalling and engineering drought hardiness in plants. Nature *410*, 327-330.

Schwartz, R., and Dayhoff, M. (1978). Matrices for detecting distant relationships. Atlas of protein sequence and structure *5*, 353-358.

Schwartz, S.H., Leon-Kloosterziel, K.M., Koornneef, M., and Zeevaart, J.A. (1997). Biochemical characterization of the aba2 and aba3 mutants in Arabidopsis thaliana. Plant physiology *114*, 161-166.

Schweighofer, A., Hirt, H., and Meskiene, I. (2004). Plant PP2C phosphatases: emerging functions in stress signaling. Trends in plant science *9*, 236-243.

Schweighofer, A., Kazanaviciute, V., Scheikl, E., Teige, M., Doczi, R., Hirt, H., Schwanninger, M., Kant, M., Schuurink, R., Mauch, F.*, et al.* (2007). The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in Arabidopsis. The Plant cell *19*, 2213-2224.

Scott, J.W., Ross, F.A., Liu, J.K., and Hardie, D.G. (2007). Regulation of AMP-activated protein kinase by a pseudosubstrate sequence on the gamma subunit. The EMBO journal *26*, 806-815.

Seki, M., Narusaka, M., Ishida, J., Nanjo, T., Fujita, M., Oono, Y., Kamiya, A., Nakajima, M., Enju, A., Sakurai, T.*, et al.* (2002). Monitoring the expression profiles of 7000 Arabidopsis genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. The Plant journal : for cell and molecular biology *31*, 279-292.

Shah, J. (2003). The salicylic acid loop in plant defense. Current opinion in plant biology *6*, 365-371.

Shakir, S.M., Bryant, K.M., Larabee, J.L., Hamm, E.E., Lovchik, J., Lyons, C.R., and Ballard, J.D. (2010). Regulatory interactions of a virulence-associated serine/threonine phosphatase-kinase pair in Bacillus anthracis. Journal of bacteriology *192*, 400-409.

Sharma, P.D., Singh, N., Ahuja, P.S., and Reddy, T.V. (2011). Abscisic acid response element binding factor 1 is required for establishment of Arabidopsis seedlings during winter. Molecular biology reports *38*, 5147-5159.

Sheen, J. (1998). Mutational analysis of protein phosphatase 2C involved in abscisic acid signal transduction in higher plants. Proceedings of the National Academy of Sciences of the United States of America *95*, 975-980.

Sheen, J. (2001). Signal Transduction in Maize and Arabidopsis Mesophyll Protoplasts. Plant physiology *127*, 1466-1475.

Shibata, N., Kagiyama, M., Nakagawa, M., Hirano, Y., and Hakoshima, T. (2010). Crystallization of the plant hormone receptors PYL9/RCAR1, PYL5/RCAR8 and PYR1/RCAR11 in the presence of (+)-abscisic acid. Acta crystallographica Section F, Structural biology and crystallization communications *66*, 456-459.

Siddappa, N.B., Avinash, A., Venkatramanan, M., and Ranga, U. (2007). Regeneration of commercial nucleic acid extraction columns without the risk of carryover contamination. Biotechniques *42*, 184-+.

Siemens, J., Keller, I., Sarx, J., Kunz, S., Schuller, A., Nagel, W., Schmulling, T., Parniske, M., and Ludwig-Muller, J. (2006). Transcriptome analysis of Arabidopsis clubroots indicate a key role for cytokinins in disease development. Molecular plant-microbe interactions : MPMI *19*, 480-494.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Soding, J.*, et al.* (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology *7*, 539.

Singh, A., Giri, J., Kapoor, S., Tyagi, A.K., and Pandey, G.K. (2010). Protein phosphatase complement in rice: genome-wide identification and transcriptional analysis under abiotic stress conditions and reproductive development. BMC genomics *11*, 435.

Sirichandra, C., Davanture, M., Turk, B.E., Zivy, M., Valot, B., Leung, J., and Merlot, S. (2010). The Arabidopsis ABA-activated kinase OST1 phosphorylates the bZIP transcription factor ABF3 and creates a 14-3-3 binding site involved in its turnover. PloS one *5*, e13935.

Sirichandra, C., Gu, D., Hu, H.C., Davanture, M., Lee, S., Djaoui, M., Valot, B., Zivy, M., Leung, J., Merlot, S*., et al.* (2009a). Phosphorylation of the Arabidopsis AtrbohF NADPH oxidase by OST1 protein kinase. FEBS letters *583*, 2982-2986.

Sirichandra, C., Wasilewska, A., Vlad, F., Valon, C., and Leung, J. (2009b). The guard cell as a single-cell model towards understanding drought tolerance and abscisic acid action. Journal of experimental botany *60*, 1439-1463.

Skriver, K., and Mundy, J. (1990). Gene expression in response to abscisic acid and osmotic stress. The Plant cell *2*, 503-512.

Soding, J. (2005). Protein homology detection by HMM-HMM comparison. Bioinformatics *21*, 951-960.

Soding, J., Biegert, A., and Lupas, A.N. (2005). The HHpred interactive server for protein homology detection and structure prediction. Nucleic acids research *33*, W244-248.

Somerville, C., and Koornneef, M. (2002). Timeline - A fortunate choice: the history of Arabidopsis as a model plant. Nature Reviews Genetics *3*, 883-889.

Song, S.K., and Clark, S.E. (2005). POL and related phosphatases are dosage-sensitive regulators of meristem and organ development in Arabidopsis. Developmental biology *285*, 272-284.

Song, S.K., Hofhuis, H., Lee, M.M., and Clark, S.E. (2008). Key divisions in the early Arabidopsis embryo require POL and PLL1 phosphatases to establish the root stem cell organizer and vascular axis. Developmental cell *15*, 98-109.

Song, S.K., Lee, M.M., and Clark, S.E. (2006). POL and PLL1 phosphatases are CLAVATA1 signaling intermediates required for Arabidopsis shoot and floral stem cells. Development *133*, 4691-4698.

Soon, F.F., Ng, L.M., Zhou, X.E., West, G.M., Kovach, A., Tan, M.H., Suino-Powell, K.M., He, Y., Xu, Y., Chalmers, M.J*., et al.* (2012a). Molecular mimicry regulates ABA signaling by SnRK2 kinases and PP2C phosphatases. Science *335*, 85-88.

Soon, F.F., Suino-Powell, K.M., Li, J., Yong, E.L., Xu, H.E., and Melcher, K. (2012b). Abscisic Acid Signaling: Thermal Stability Shift Assays as Tool to Analyze Hormone Perception and Signal Transduction. PloS one *7*.

Stark, C., Breitkreutz, B.J., Reguly, T., Boucher, L., Breitkreutz, A., and Tyers, M. (2006). BioGRID: a general repository for interaction datasets. Nucleic acids research *34*, D535-539.

Stone, S.L., Williams, L.A., Farmer, L.M., Vierstra, R.D., and Callis, J. (2006). KEEP ON GOING, a RING E3 ligase essential for Arabidopsis growth and development, is involved in abscisic acid signaling. The Plant cell *18*, 3415-3428.

Sueyoshi, N., Nimura, T., Onouchi, T., Baba, H., Takenaka, S., Ishida, A., and Kameshita, I. (2012). Functional processing of nuclear Ca2+/calmodulin-dependent protein kinase phosphatase (CaMKP-N): evidence for a critical role of proteolytic processing in the regulation of its catalytic activity, subcellular localization and substrate targeting in vivo. Archives of biochemistry and biophysics *517*, 43-52.

Sun, H., Kim, M.K., Pulla, R.K., Kim, Y.J., and Yang, D.C. (2010). Isolation and expression analysis of a novel major latex-like protein (MLP151) gene from Panax ginseng. Molecular biology reports *37*, 2215-2222.

Suzuki, H., Xia, Y., Cameron, R., Shadle, G., Blount, J., Lamb, C., and Dixon, R.A. (2004). Signals for local and systemic responses of plants to pathogen attack. Journal of experimental botany *55*, 169-179.

Szostkiewicz, I. (2010). Regulatory components of the abscisic acid receptors in Arabidopsis thaliana (Freising: Technische Universität München), pp. 148.

Szostkiewicz, I., Richter, K., Kepka, M., Demmel, S., Ma, Y., Korte, A., Assaad, F.F., Christmann, A., and Grill, E. (2010). Closely related receptor complexes differ in their ABA selectivity and sensitivity. The Plant journal : for cell and molecular biology *61*, 25-35.

Tada, Y., Nimura, T., Sueyoshi, N., Ishida, A., Shigeri, Y., and Kameshita, I. (2006). Mutational analysis of Ca(2+)/calmodulin-dependent protein kinase phosphatase (CaMKP). Archives of biochemistry and biophysics *452*, 174-185.

Tahtiharju, S., and Palva, T. (2001). Antisense inhibition of protein phosphatase 2C accelerates cold acclimation in Arabidopsis thaliana. The Plant journal : for cell and molecular biology *26*, 461-470.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution *28*, 2731-2739.

Tirode, F., Malaguti, C., Romero, F., Attar, R., Camonis, J., and Egly, J.M. (1997). A conditionally expressed third partner stabilizes or prevents the formation of a transcriptional activator in a three-hybrid system. The Journal of biological chemistry *272*, 22995-22999.

Ton, J., Flors, V., and Mauch-Mani, B. (2009). The multifaceted role of ABA in disease resistance. Trends in plant science *14*, 310-317.

Toufighi, K., Brady, S.M., Austin, R., Ly, E., and Provart, N.J. (2005). The Botany Array Resource: e-Northerns, Expression Angling, and promoter analyses. The Plant journal : for cell and molecular biology *43*, 153-163.

Tripathi, V., Parasuraman, B., Laxmi, A., and Chattopadhyay, D. (2009). CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plants. The Plant journal : for cell and molecular biology *58*, 778-790.

Truman, W., Bennett, M.H., Kubigsteltig, I., Turnbull, C., and Grant, M. (2007). Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. Proceedings of the National Academy of Sciences of the United States of America *104*, 1075-1080.

Tsou, P.L., Lee, S.Y., Allen, N.S., Winter-Sederoff, H., and Robertson, D. (2012). An ER-targeted calcium-binding peptide confers salt and drought tolerance mediated by CIPK6 in Arabidopsis. Planta *235*, 539-552.

Ubersax, J.A., and Ferrell, J.E., Jr. (2007). Mechanisms of specificity in protein phosphorylation. Nature reviews Molecular cell biology *8*, 530-541.

Umbrasaite, J., Schweighofer, A., Kazanaviciute, V., Magyar, Z., Ayatollahi, Z., Unterwurzacher, V., Choopayak, C., Boniecka, J., Murray, J.A., Bogre, L*., et al.* (2010). MAPK phosphatase AP2C3 induces ectopic proliferation of epidermal cells leading to stomata development in Arabidopsis. PloS one *5*, e15357.

Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2010). Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. Plant & cell physiology *51*, 1821-1839.

Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K., Ishihama, Y., Hirayama, T., and Shinozaki, K. (2009). Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America *106*, 17588-17593.

Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2004). SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America *101*, 17306-17311.

Uno, Y., Furihata, T., Abe, H., Yoshida, R., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2000). Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. Proceedings of the National Academy of Sciences of the United States of America *97*, 11632-11637.

Valdes, A.E., Overnas, E., Johansson, H., Rada-Iglesias, A., and Engstrom, P. (2012). The homeodomain-leucine zipper (HD-Zip) class I transcription factors ATHB7 and ATHB12 modulate abscisic acid signalling by regulating protein phosphatase 2C and abscisic acid receptor gene activities. Plant molecular biology.

van Ittersum, M.K., Cassman, K.G., Grassini, P., Wolf, J., Tittonell, P., and Hochman, Z. (2013). Yield gap analysis with local to global relevance. Field Crops Research *143*, 4-17.

Vlad, F., Droillard, M.J., Valot, B., Khafif, M., Rodrigues, A., Brault, M., Zivy, M., Rodriguez, P.L., Merlot, S., and Lauriere, C. (2010). Phospho-site mapping, genetic and in planta activation studies reveal key aspects of the different phosphorylation mechanisms involved in activation of SnRK2s. The Plant journal : for cell and molecular biology *63*, 778-790.

Vlad, F., Rubio, S., Rodrigues, A., Sirichandra, C., Belin, C., Robert, N., Leung, J., Rodriguez, P.L., Lauriere, C., and Merlot, S. (2009). Protein phosphatases 2C regulate the activation of the Snf1-related kinase OST1 by abscisic acid in Arabidopsis. The Plant cell *21*, 3170-3184.

Vlot, A.C., Dempsey, D.A., and Klessig, D.F. (2009). Salicylic Acid, a multifaceted hormone to combat disease. Annual review of phytopathology *47*, 177-206.

Vranova, E., Tahtiharju, S., Sriprang, R., Willekens, H., Heino, P., Palva, E.T., Inze, D., and Van Camp, W. (2001). The AKT3 potassium channel protein interacts with the AtPP2CA protein phosphatase 2C. Journal of experimental botany *52*, 181-182.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C., and Zhang, S. (2007). Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. The Plant cell *19*, 63-73.

Wang, M., Oppedijk, B.J., Lu, X., Van Duijn, B., and Schilperoort, R.A. (1996). Apoptosis in barley aleurone during germination and its inhibition by abscisic acid. Plant molecular biology *32*, 1125-1134.

Wang, R.S., Pandey, S., Li, S., Gookin, T.E., Zhao, Z., Albert, R., and Assmann, S.M. (2011). Common and unique elements of the ABA-regulated transcriptome of Arabidopsis guard cells. BMC genomics *12*, 216.

Wang, W.M., Ma, X.F., Zhang, Y., Luo, M.C., Wang, G.L., Bellizzi, M., Xiong, X.Y., and Xiao, S.Y. (2012). PAPP2C Interacts with the Atypical Disease Resistance Protein RPW8.2 and Negatively Regulates Salicylic Acid-Dependent Defense Responses in Arabidopsis. Molecular plant *5*, 1125-1137.

Wang, Y., Li, L., Ye, T., Lu, Y., Chen, X., and Wu, Y. (2013). The inhibitory effect of ABA on floral transition is mediated by ABI5 in Arabidopsis. Journal of experimental botany *64*, 675-684.

Weiner, J.J., Peterson, F.C., Volkman, B.F., and Cutler, S.R. (2010). Structural and functional insights into core ABA signaling. Current opinion in plant biology *13*, 495-502.

Wernimont, A.K., Artz, J.D., Finerty, P., Jr., Lin, Y.H., Amani, M., Allali-Hassani, A., Senisterra, G., Vedadi, M., Tempel, W., Mackenzie, F.*, et al.* (2010). Structures of apicomplexan calcium-dependent protein kinases reveal mechanism of activation by calcium. Nature structural & molecular biology *17*, 596-601.

Widjaja, I., Lassowskat, I., Bethke, G., Eschen-Lippold, L., Long, H.H., Naumann, K., Dangl, J.L., Scheel, D., and Lee, J. (2010). A protein phosphatase 2C, responsive to the bacterial effector AvrRpm1 but not to the AvrB effector, regulates defense responses in Arabidopsis. The Plant journal : for cell and molecular biology *61*, 249-258.

Wu, Y., Sanchez, J.P., Lopez-Molina, L., Himmelbach, A., Grill, E., and Chua, N.H. (2003). The abi1-1 mutation blocks ABA signaling downstream of cADPR action. The Plant journal : for cell and molecular biology *34*, 307-315.

Wu, Y., Zhang, D., Chu, J.Y., Boyle, P., Wang, Y., Brindle, I.D., De Luca, V., and Despres, C. (2012). The Arabidopsis NPR1 protein is a receptor for the plant defense hormone salicylic acid. Cell reports *1*, 639-647.

Xia, Y., Yang, Q., Gong, X., Ye, F., and Liou, Y.C. (2011). Dose-dependent mutual regulation between Wip1 and p53 following UVC irradiation. The international journal of biochemistry & cell biology *43*, 535-544.

Xie, T., Ren, R., Zhang, Y.Y., Pang, Y., Yan, C., Gong, X., He, Y., Li, W., Miao, D., Hao, Q.*, et al.* (2012). Molecular mechanism for inhibition of a critical component in the Arabidopsis thaliana abscisic acid signal transduction pathways, SnRK2.6, by protein phosphatase ABI1. The Journal of biological chemistry *287*, 794-802.

Xu, J., Li, H.D., Chen, L.Q., Wang, Y., Liu, L.L., He, L., and Wu, W.H. (2006). A protein kinase, interacting with two calcineurin B-like proteins, regulates K+ transporter AKT1 in Arabidopsis. Cell *125*, 1347-1360.

Xu, J., Tian, Y.S., Peng, R.H., Xiong, A.S., Zhu, B., Jin, X.F., Gao, F., Fu, X.Y., Hou, X.L., and Yao, Q.H. (2010). AtCPK6, a functionally redundant and positive regulator involved in salt/drought stress tolerance in Arabidopsis. Planta *231*, 1251-1260.

Xue, S., Hu, H., Ries, A., Merilo, E., Kollist, H., and Schroeder, J.I. (2011). Central functions of bicarbonate in S-type anion channel activation and OST1 protein kinase in CO2 signal transduction in guard cell. The EMBO journal *30*, 1645-1658.

Xue, T., Wang, D., Zhang, S., Ehlting, J., Ni, F., Jakab, S., Zheng, C., and Zhong, Y. (2008). Genome-wide and expression analysis of protein phosphatase 2C in rice and Arabidopsis. BMC genomics *9*, 550.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1993). Arabidopsis DNA encoding two desiccation-responsive rd29 genes. Plant physiology *101*, 1119-1120.

Yang, M., Song, S., Liu, G., Chen, K., Tian, X., Zhao, Z.J., Hu, S., and Yu, J. (2010). A comprehensive analysis of protein phosphatases in rice and Arabidopsis. Plant Systematics and Evolution *289*, 111-126.

Yang, Y. (2003). Signal Transduction of abscisic acid in Arabidopsis thaliana: Identification and characterisation of protein interaction partners of ABI2. In Lehrstuhl für Botanik (München: Technische Universität München), pp. 188.

Yang, Y., Sulpice, R., Himmelbach, A., Meinhard, M., Christmann, A., and Grill, E. (2006). Fibrillin expression is regulated by abscisic acid response regulators and is involved in abscisic acid-mediated photoprotection. Proceedings of the National Academy of Sciences of the United States of America *103*, 6061-6066.

Yin, P., Fan, H., Hao, Q., Yuan, X., Wu, D., Pang, Y., Yan, C., Li, W., Wang, J., and Yan, N. (2009). Structural insights into the mechanism of abscisic acid signaling by PYL proteins. Nature structural & molecular biology *16*, 1230-1236.

Yoo, S.D., Cho, Y.H., and Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nature protocols *2*, 1565-1572.

Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F., and Shinozaki, K. (2006a). The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. The Journal of biological chemistry *281*, 5310-5318.

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2010). AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. The Plant journal : for cell and molecular biology *61*, 672-685.

Yoshida, T., Nishimura, N., Kitahata, N., Kuromori, T., Ito, T., Asami, T., Shinozaki, K., and Hirayama, T. (2006b). ABA-hypersensitive germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs. Plant physiology *140*, 115-126.

Young, C., Mapes, J., Hanneman, J., Al-Zarban, S., and Ota, I. (2002). Role of Ptc2 Type 2C Ser/Thr Phosphatase in Yeast High-Osmolarity Glycerol Pathway Inactivation. Eukaryotic cell *1*, 1032-1040.

Yu, J., Hu, S., Wang, J., Wong, G.K., Li, S., Liu, B., Deng, Y., Dai, L., Zhou, Y., Zhang, X.*, et al.* (2002). A draft sequence of the rice genome (Oryza sativa L. ssp. indica). Science *296*, 79-92.

Yu, Y., Xia, X., Yin, W., and Zhang, H. (2007). Comparative genomic analysis of CIPK gene family in Arabidopsis and Populus. Plant Growth Regulation *52*, 101-110.

Yunta, C., Martinez-Ripoll, M., Zhu, J.K., and Albert, A. (2011). The structure of Arabidopsis thaliana OST1 provides insights into the kinase regulation mechanism in response to osmotic stress. Journal of molecular biology *414*, 135-144.

Zander, M., La Camera, S., Lamotte, O., Metraux, J.P., and Gatz, C. (2010). Arabidopsis thaliana class-II TGA transcription factors are essential activators of jasmonic acid/ethylene-induced defense responses. The Plant journal : for cell and molecular biology *61*, 200-210.

Zentella, R., Zhang, Z.L., Park, M., Thomas, S.G., Endo, A., Murase, K., Fleet, C.M., Jikumaru, Y., Nambara, E., Kamiya, Y.*, et al.* (2007). Global analysis of della direct targets in early gibberellin signaling in Arabidopsis. The Plant cell *19*, 3037-3057.

Zhang, J., Li, X., He, Z., Zhao, X., Wang, Q., Zhou, B., Yu, D., Huang, X., Tang, D., Guo, X.*, et al.* (2012a). Molecular character of a phosphatase 2C (PP2C) gene relation to stress tolerance in Arabidopsis thaliana. Molecular biology reports.

Zhang, J.H., Schurr, U., and Davies, W.J. (1987). Control of Stomatal Behavior by Abscisic-Acid Which Apparently Originates in the Roots. Journal of experimental botany *38*, 1174-1181.

Zhang, K., Xia, X., Zhang, Y., and Gan, S.S. (2012b). An ABA-regulated and Golgi-localized protein phosphatase controls water loss during leaf senescence in Arabidopsis. The Plant journal : for cell and molecular biology *69*, 667-678.

Zhang, X., Henriques, R., Lin, S.S., Niu, Q.W., and Chua, N.H. (2006). Agrobacterium-mediated transformation of Arabidopsis thaliana using the floral dip method. Nature protocols *1*, 641-646.

Zhao, R., Wang, X.F., and Zhang, D.P. (2011). CPK12: A Ca2+-dependent protein kinase balancer in abscisic acid signaling. Plant signaling & behavior *6*, 1687-1690.

Zheng, L., Baumann, U., and Reymond, J.L. (2004). An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucleic acids research *32*, e115.

Zhu, J.K. (2002). Salt and drought stress signal transduction in plants. Annual review of plant biology *53*, 247-273.

Zhu, S.Y., Yu, X.C., Wang, X.J., Zhao, R., Li, Y., Fan, R.C., Shang, Y., Du, S.Y., Wang, X.F., Wu, F.Q*., et al.* (2007). Two calcium-dependent protein kinases, CPK4 and CPK11, regulate abscisic acid signal transduction in Arabidopsis. The Plant cell *19*, 3019-3036.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G., and Boller, T. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. Nature *428*, 764-767.

Zou, J.J., Wei, F.J., Wang, C., Wu, J.J., Ratnasekera, D., Liu, W.X., and Wu, W.H. (2010). Arabidopsis calcium-dependent protein kinase CPK10 functions in abscisic acid- and Ca2+-mediated stomatal regulation in response to drought stress. Plant physiology *154*, 1232-1243.

Zuckerkandl, E., and Pauling, L. (1965). Evolutionary divergence and convergence in proteins. Evolving genes and proteins *97*, 166.

6 APPENDIX

6.1 Strains used in this work

Strainlist #	Organism	Strain	Vector/Plasmid	Resistance
427	EC	DH5α	pBI121AscI (original)	Kan
720	EC	DH5α	pSK 35S S1 EYFP	Amp
883	EC	DH5α	pSK 35S Ω GUS	Amp
1864	EC	DH5α	pGAD424 empty	Amp
2066	EC	DH5α	pGBT9 ABI2	Amp
2240	EC	DH5α	pBridge At1g78200 clade F PP2C	Amp
2244	EC	DH5α	pBridge At2g25070	Amp
2462	EC	XL1 blue	pGAD_ABI2	Amp
2463	EC	XL1 blue	pGAD_ABI1	Amp
2586	Y	AH109	empty strain for competent cells	
2970	EC	DH5α	empty strain for competent cells	
2986	EC	DH5α	pGAD SnRK2.2	Amp
3041	EC	DH5α	pSK pRD29B::LUC new	Amp
3076	EC	DH5α	pBridge-RCAR6 clone 1	Amp
3078	EC	DH5α	pBridge-RCAR13 clone 1	Amp
3080	EC	DH5α	pBridge-RCAR14 clone 1	Amp
3212	EC	XL1 blue	pGBT SnRK2.2	Amp
3214	EC	XL1 blue	pGAD SnRK2.2	Amp
3215	EC	XL1 blue	pGAD OST1	Amp
3216	EC	XL1 blue	pBridge OST1	Amp
3218	EC	DH5α	pBridge empty vector	Amp
3227	EC	DH5α	pGAD 1g78200	Amp
3229	EC	DH5α	pGAD 2g25070	Amp
3295	EC	XL1 blue	pGAD424 At1g14950	Amp
3297	EC	XL1 blue	pGAD424 At1g23120	Amp
3299	EC	XL1 blue	pGAD424 At1g24020	Amp
3301	EC	XL1 blue	pGAD424 At1g35310	Amp
3303	EC	XL1 blue	pGAD424 At1g70830.1	Amp
3305	EC	XL1 blue	pGAD424 At4g23670	Amp
3307	EC	XL1 blue	pBridge At1g14950	Amp
3309	EC	XL1 blue	pBridge At1g23120	Amp
3311	EC	XL1 blue	pBridge At1g24020	Amp
3313	EC	XL1 blue	pBridge At1g35310	Amp
3315	EC	XL1 blue	pBridge At1g70830.1	Amp
3317	EC	XL1 blue	pBridge At4g23670	Amp
3331	Y	AH109	empty strain for competent cells	
3333	EC	DH5α	empty strain for competent cells	
3340	EC	XL1 blue	empty strain for competent cells	Tet
3463	EC	DH5α	pGAD 5g02760	Amp
3465	EC	DH5α	pBridge 5g02760	Amp
3467	EC	DH5α	pGAD 3g16560	Amp
3469	EC	DH5α	pBridge 3g16560	Amp
3471	EC	DH5α	pGAD 5g27930	Amp
3473	EC	DH5α	pBridge 5g27930	Amp
3475	EC	DH5α	pGAD 5g66720	Amp
3477	EC	DH5α	pBridge 5g66720	Amp
3479	EC	DH5α	pGAD 5g66080	Amp
3481	EC	DH5α	pBridge 5g66080	Amp
3483	EC	DH5α	pGAD 2g33700	Amp
3485	EC	DH5α	pBridge 2g33700	Amp
3487	EC	DH5α	pGAD 1g79630	Amp
3489	EC	DH5α	pBridge 1g79630	Amp
3491	EC	DH5α	pGAD 1g07630	Amp
3493	EC	DH5α	pBridge 1g07630	Amp
3546	EC	DH5α	pBridge 3g11410 PP2C	Amp
3548	EC	DH5α	pGAD 3g11410 PP2C	Amp

3550	EC	DH5α	pBridge 2g29380 PP2C	Amp
3552	EC	DH5α	pGAD 2g29380 PP2C	Amp
3554	EC	DH5α	pBridge 1g48040 PP2C	Amp
3556	EC	DH5α	pGAD 1g48040 PP2C	Amp
3558	EC	DH5α	pBridge 2g20050 PP2C	Amp
3560	EC	DH5α	pGAD 2g20050 PP2C	Amp
3562	EC	DH5α	pBridge 2g40180 PP2C	Amp
3564	EC	DH5α	pGAD 2g40180 PP2C	Amp
3566	EC	DH5α	pBridge 4g31750 PP2C	Amp
3568	EC	DH5α	pGAD 4g31750 PP2C	Amp
3570	EC	DH5α	pBridge 1g07160 PP2C	Amp
3572	EC	DH5α	pGAD 1g07160 PP2C	Amp
3590	EC	XL1 blue	pGAD ABI5	Amp
3606	EC	XL1 blue	pGBT SnRK2.3	Amp
3638	EC	DH5α	pGBT CIPK1	Amp
3639	EC	DH5α	pGBT CIPK2	Amp
3640	EC	DH5α	pGBT CIPK3	Amp
3641	EC	DH5α	pGBT CIPK4	Amp
3642	EC	DH5α	pGBT CIPK5	Amp
3643	EC	DH5α	pGBT CIPK6	Amp
3644	EC	DH5α	pGBT CIPK7	Amp
3645	EC	DH5α	pGBT CIPK8	Amp
3646	EC	DH5α	pGBT CIPK9	Amp
3647	EC	DH5α	pGBT CIPK10	Amp
3648	EC	DH5α	pGBT CIPK11	Amp
3649	EC	DH5α	pGBT CIPK12	Amp
3650	EC	DH5α	pGBT CIPK13	Amp
3651	EC	DH5α	pGBT CIPK14	Amp
3652	EC	DH5α	pGBT CIPK15	Amp
3653	EC	DH5α	pGBT CIPK16	Amp
3654	EC	DH5α	pGBT CIPK17	Amp
3655	EC	DH5α	pGBT CIPK18	Amp
3656	EC	DH5α	pGBT CIPK19	Amp
3657	EC	DH5α	pGBT CIPK20	Amp
3658	EC	DH5α	pGBT CIPK21	Amp
3659	EC	DH5α	pGBT CIPK22	Amp
3660	EC	DH5α	pGBT CIPK24	Amp
3661	EC	DH5α	pGBT CIPK25	Amp
3721	EC	XL1 blue	pGAD 1g47380 PP2C #5	Amp
3841	EC	DH5α	pGBT CIPK23	Amp
3844	EC	XL1 blue	pBridge RCAR4	Amp
3858	EC	XL1 blue	pBridge GCA2 vk	Amp
3883	EC	DH5α	pBridge RCAR12	Amp
3887	EC	DH5α	pBridge RCAR9	Amp
3891	EC	DH5α	pBridge RCAR10	Amp
3895	EC	DH5α	pBridge RCAR11	Amp
3899	EC	DH5α	pGAD At1g07430	Amp
3903	EC	DH5α	pGAD At5g59220	Amp
3962	EC	XL1 blue	pBridge RCAR7	Amp
3968	EC	DH5α	pBridge RCAR2	Amp
3972	EC	DH5α	pBridge RCAR8	Amp
3976	EC	DH5α	pBridge RCAR5	Amp
3986	EC	XL1 blue	pBI221 At1g16220 Col	Amp
4062	EC	DH5α	pBridge At1g16220	Amp
4063	EC	DH5α	pSASFa 35S PP2CA	Amp
4064	EC	DH5α	pSASFa 35S HAI3	Amp
4065	EC	DH5α	pSASFa	Amp
4072	EC	DH5α	pSASFa RCAR5	Amp
4073	EC	DH5α	pSASFa RCAR8	Amp
4074	EC	DH5α	pSASFa RCAR12	Amp
4075	EC	DH5α	pSASFa HAI1	Amp
4076	EC	DH5α	pSASFa HAI2	Amp

4095	EC	DH5α	pSASFa ABI1	Amp
4096	EC	DH5α	pSASFa ABI2	Amp
4097	EC	DH5α	pSASFa RCAR1	Amp
4098	EC	DH5α	pSASFa RCAR3	Amp
4099	EC	DH5α	pSASFa RCAR11	Amp
4331	EC	DH5α	pGAD424 HAB1 (neu)	Amp
4333	EC	DH5α	pGAD424 HAB2 (neu)	Amp
4335	EC	DH5α	pSASFb	Amp
4353	EC	DH5α	pSK AscI CIPK2	Amp
4354	EC	DH5α	pSK AscI CIPK5	Amp
4355	EC	DH5α	pSK AscI CIPK8	Amp
4356	EC	DH5α	pSK AscI CIPK9	Amp
4357	EC	DH5α	pSK AscI CIPK12	Amp
4358	EC	DH5α	pSK AscI CIPK14	Amp
4359	EC	DH5α	pSK AscI CIPK15	Amp
4360	EC	DH5α	pSK AscI CIPK16	Amp
4361	EC	DH5α	pSK AscI CIPK18	Amp
4362	EC	DH5α	pSK AscI CIPK19	Amp
4363	EC	DH5α	pSK AscI CIPK20	Amp
4364	EC	DH5α	pSK AscI CIPK21	Amp
4365	EC	DH5α	pSK AscI CIPK24	Amp
4366	EC	DH5α	pSK AscI CIPK3	Amp
4367	EC	DH5α	pSK AscI CIPK11	Amp
4368	EC	DH5α	pSK AscI CIPK13	Amp
4388	EC	DH5α	pSK35S RCAR2	Amp
4389	EC	DH5α	pSK35S RCAR4	Amp
4390	EC	DH5α	pSK35S RCAR6	Amp
4391	EC	DH5α	pSK35S RCAR7	Amp
4392	EC	DH5α	pSK35S RCAR9	Amp
4393	EC	DH5α	pSK35S RCAR10	Amp
4394	EC	DH5α	pSK35S RCAR13	Amp
4395	EC	XL1 blue	pSK35S RCAR14	Amp
4396	EC	DH5α	pSK AscI CIPK2 CA	Amp
4397	EC	DH5α	pSK AscI CIPK9 CA	Amp
4398	EC	DH5α	pSK AscI CIPK14 CA	Amp
4399	EC	DH5α	pSK AscI CIPK15 CA	Amp
4400	EC	DH5α	pSK AscI CIPK21 CA	Amp
4401	EC	DH5α	pSK AscI CIPK24 CA	Amp
4411	EC	DH5α	pSK AscI 35S CIPK8 (CA)	Amp
4412	EC	DH5α	pSK AscI 35S CIPK12 (CA)	Amp
4413	EC	DH5α	pSK AscI 35S CIPK18 (CA)	Amp
4414	EC	DH5α	pSK AscI 35S CIPK20 (CA)	Amp
4421	EC	DH5α	pSK AscI35S CIPK3 (CA)	Amp
4422	EC	DH5α	pSK AscI35S CIPK5 (CA)	Amp
4423	EC	DH5α	pSK AscI35S CIPK19 (CA)	Amp
4428	EC	DH5α	pSK AscI 35S CIPK13 (CA)	Amp
4431	EC	DH5α	pSK AscI 35S CIPK11 (CA)	Amp
4453	EC	DH5α	pSK AscI 35S CPK11	Amp
4454	EC	DH5α	pSK AscI 35S CIPK16 (CA)	Amp
4484	EC	XL1 blue	pGAD MLP34 CDS	Amp
4485	EC	XL1 blue	pBridge MLP34 CDS	Amp
4524	EC	DH5α	pBridge GCA2 VL (Ler)	Amp
4525	EC	DH5α	pBridge GCA2 VK	Amp
4526	EC	DH5α	pBridge GCA2 VLTK	Amp
4527	EC	DH5α	pBridge GCA2 VKTK	Amp
4528	EC	DH5α	pBridge CPK21 VL (Col wt)	Amp
4529	EC	DH5α	pBridge CPK21 VLTK	Amp
4530	EC	DH5α	pBridge CPK21 VK	Amp
4531	EC	DH5α	pBridge CPK21 VKTK	Amp
4568	EC	DH5α	pBridge GCA2_RCAR1	Amp
4569	EC	DH5α	pBridge GCA2_RCAR9	Amp
4570	EC	DH5α	pBridge GCA2_RCAR11	Amp

4571	EC	DH5α	pBridge GCA2_SnRK2.2	Amp
4572	EC	DH5α	pBridge GCA2 VK_RCAR1	Amp
4573	EC	DH5α	pBridge GCA2 VK_RCAR9	Amp
4574	EC	DH5α	pBridge GCA2 VK_RCAR11	Amp
4575	EC	DH5α	pBridge GCA2 VK_SnRK2.2	Amp
4578	EC	DH5α	pSASFa PP2C 5g27930	Amp
4579	EC	DH5α	pSASFa PP2C 1g79630	Amp
4580	EC	DH5α	pSASFa PP2C 1g16220	Amp
4646	EC	DH5α	pBridge CPK21 VK/RCAR1	Amp
4647	EC	DH5α	pBridge CPK21 VK/RCAR9	Amp
4648	EC	DH5α	pBridge CPK21 VK/RCAR11	Amp
4649	EC	DH5α	pBridge CPK21 VK/SnRK2.2	Amp
4650	EC	DH5α	pBridge CPK21 VKTK/RCAR1	Amp
4651	EC	DH5α	pBridge CPK21 VKTK/RCAR9	Amp
4652	EC	DH5α	pBridge CPK21 VKTK/RCAR11	Amp
4653	EC	DH5α	pBridge CPK21 VKTK/SnRK2.2	Amp
4654	EC	DH5α	pSASFa AHG1 genom	Amp
4655	EC	DH5α	pBridge CPK21 VK	Amp
4656	EC	DH5α	pBridge CPK21 VKTK	Amp
4657	EC	DH5α	pGAD tMLP34	Amp
4658	EC	DH5α	pBridge tMLP34	Amp
4659	EC	DH5α	pBridge GCA2 VKTK/RCAR1	Amp
4660	EC	DH5α	pBridge GCA2 VKTK/RCAR9	Amp
4661	EC	DH5α	pBridge GCA2 VKTK/RCAR11	Amp
4662	EC	DH5α	pBridge GCA2 VKTK/SnRK2.2	Amp
4663	EC	DH5α	pBridge GCA2 VLTK/RCAR1	Amp
4664	EC	DH5α	pBridge CPK21/RCAR1	Amp
4665	EC	DH5α	pBridge CPK21 TK/RCAR1	Amp
4666	EC	DH5α	pBridge GCA2 VLTK/SnRK2.2	Amp
4667	EC	DH5α	pBridge CPK21/SnRK2.2	Amp
4668	EC	DH5α	pBridge CPK21 TK/SnRK2.2	Amp
4669	EC	DH5α	pBridge GCA2 VLTK/RCAR11	Amp
4670	EC	DH5α	pBridge CPK21/RCAR11	Amp
4671	EC	DH5α	pBridge CPK21 TK/RCAR11	Amp
4672	EC	DH5α	pBridge GCA2 VLTK/RCAR9	Amp
4673	EC	DH5α	pBridge CPK21/RCAR9	Amp
4674	EC	DH5α	pBridge CPK21 TK/RCAR9	Amp
4735	EC	DH5α	pSASFa t34 (tMLP34)	Amp
4736	EC	DH5α	pSK pPR1(L)::LUC	Amp
4836	EC	DH5α	pBridge GCA2_SnRK2.6	Amp
4837	EC	DH5α	pBridge GCA2 VK_SnRK2.6	Amp
4838	EC	DH5α	pBridge GCA2 TK_SnRK2.6	Amp
4839	EC	DH5α	pBridge GCA2 VKTK_SnRK2.6	Amp
4840	EC	DH5α	pBridge CPK21_SnRK2.6	Amp
4841	EC	DH5α	pBridge CPK21 TK_SnRK2.6	Amp
4842	EC	DH5α	pBridge CPK21 VK_SnRK2.6	Amp
4843	EC	DH5α	pBridge CPK21 VKTK_SnRK2.6	Amp
4850	EC	DH5α	pQE70 tMLP34	Amp
4851	EC	DH5α	pSASFa WIN2	Amp
4854	EC	DH5α	pBridge GCA2_SnRK2.6 TK	Amp
4855	EC	DH5α	pBridge GCA2 VK_SnRK2.6 TK	Amp
4856	EC	DH5α	pBridge GCA2 TK_SnRK2.6 TK	Amp
4857	EC	DH5α	pBridge GCA2 VKTK_SnRK2.6 TK	Amp
4858	EC	DH5α	pBridge CPK21_SnRK2.6 TK	Amp
4859	EC	DH5α	pBridge CPK21 TK_SnRK2.6 TK	Amp
4860	EC	DH5α	pBridge CPK21 VK_SnRK2.6 TK	Amp
4861	EC	DH5α	pBridge CPK21 VKTK_SnRK2.6 TK	Amp
4868	EC	DH5α	pBridge RCAR1_GCA2 VK	Amp
4869	EC	DH5α	pBridge RCAR1_GCA2 VKTK	Amp
4870	EC	DH5α	pBridge RCAR9_GCA2 VK	Amp
4871	EC	DH5α	pBridge RCAR9_GCA2 VKTK	Amp
4872	EC	DH5α	pBridge RCAR11_GCA2 VK	Amp

4873	EC	DH5α	pBridge RCAR11_GCA2 VKTK	Amp
4874	EC	DH5α	pBridge SnRK2.2_GCA2 VK	Amp
4875	EC	DH5α	pBridge SnRK2.2_GCA2 VKTK	Amp
4876	EC	DH5α	pBridge SnRK2.3_GCA2 VK	Amp
4877	EC	DH5α	pBridge SnRK2.3_GCA2 VKTK	Amp
4878	EC	DH5α	pBridge SnRK2.6_GCA2 VK	Amp
4879	EC	DH5α	pBridge SnRK2.6_GCA2 VKTK	Amp
4880	EC	DH5α	pGAD AHG1	Amp
4890	EC	DH5α	pSASFa MLP34	Amp
4945	EC	DH5α	pSASFa MLP28	Amp
4972	EC	DH5α	pBI121 35S CIPK11 CA	Kan
4973	EC	DH5α	pBI121 35S CIPK20	Kan
4974	EC	DH5α	pBI121 35S CIPK11	Kan
4975	EC	DH5α	pBI121 35S CIPK14 CA	Kan
4976	EC	DH5α	pBI121 35S CIPK20 CA	Kan
4977	EC	DH5α	pBI121 35S CIPK2 CA	Kan
4978	EC	DH5α	pBI121 35S CIPK14	Kan
4979	EC	DH5α	pBI121 35S CIPK2	Kan
4980	EC	DH5α	pBI121 35S empty Ter	Kan
5007	EC	XL1 blue	pGAD_HopW1-1_fl	Amp
5008	EC	XL1 blue	pGAD_HopW1-1_truncated	Amp
5011	Atu	GV3101	pBI121 35S CIPK2 wt	Rif/Kan
5012	Atu	GV3101	pBI121 35S CIPK2 CA	Rif/Kan
5013	Atu	GV3101	pBI121 35S CIPK11 wt	Rif/Kan
5014	Atu	GV3101	pBI121 35S CIPK11 CA	Rif/Kan
5015	Atu	GV3101	pBI121 35S CIPK14 wt	Rif/Kan
5016	Atu	GV3101	pBI121 35S CIPK14 CA	Rif/Kan
5017	Atu	GV3101	pBI121 35S CIPK20 wt	Rif/Kan
5018	Atu	GV3101	pBI121 35S CIPK20 CA	Rif/Kan
5019	Atu	GV3101	pBI121 35S empty Ter	Rif/Kan
5196	EC	DH5α	pFRK1::LUC	Amp
5197	EC	DH5α	pNHL1::LUC	Amp
5198	EC	DH5α	pPHI1::LUC	Amp
5199	EC	DH5α	pUBQ10::GUS	Amp
5216	EC	DH5α	pSASFa MLP C	Amp
5217	EC	DH5α	pSASFa MLP D	Amp
5218	EC	DH5α	pSASFa MLP A	Amp
5219	EC	DH5α	pSASFa MLP F	Amp
5221	EC	DH5α	pBridge ABI5	Amp
5227	EC	DH5α	pSASFa MLP I	Amp
5229	EC	DH5α	pSASFa MLP31	Amp
5230	EC	DH5α	pSASFa ABF3	Amp
5231	EC	DH5α	pSASFa MLP G	Amp
5232	EC	DH5α	pSASFa MLP H	Amp

6.2 Oligonucleotides used in this work

#	Purpose	Gene	Name	Nucleotide-Sequence (5' -> 3')
319	Y2H	At5g59220	5g59220 EcoRI f	gagtcGAATTCATGGCTGAGATTTGTTACGAGAACG
320	Y2H	At5g59220	5g59220 BglII r	ctcgaAGATCTCTACGTGTCTCGTCGTAGATCAACC
321	Y2H	At4g08260	4g08260 EcoRI f	gagtcGAATTCATGGAGGATCGGTTCTCCGCTATC
322	Y2H	At4g08260	4g08260 BclI r	ctcgaTGATCACTATACGAACTGGCGCAATGGGATC
323	Y2H	At1g78200	1g78200 EcoRI f	gagtcGAATTCATGCCCAAGATCTGCTGCTCTCGTTCC
324	Y2H	At1g78200	1g78200 BamHI r	ctcgaG GATCCTCATCTAAACCGGACAACAATGC
325	Y2H	At1g72770	1g72770 EcoRI f	gagtcGAATTCATGGAGGAGATGACTCCCGCAGTTG
326	Y2H	At1g72770	1g72770 BamHI r	ctgaGGATCCTCAGGTTCTGGTCTTGAACTTTC
327	Y2H	At2g25070	2g25070 EcoRI f	gagtcGAATTCATGGGTACATACCTAAGTTCTCC
328	Y2H	At2g25070	2g25070 BamHI r	ctcgaGGATCCCTAGCTTGATGAGCTCGGCTCATCT
386	Y2H	At4g17870	At4g17870-Eco-f	gagtcGAATTCATGCCTTCGGAGTTAACACCAGAAG
387	Y2H	At4g17870	At4g17870-Bam-r	gactcGGATCCTCACGTCACCTGAGAACCACTTCC

388	Y2H	At5g05440 RCAR8	At5g05440-EcoRI-f	gagtcGAATTCATGAGGTCACCGGTGCAACTCC
389	Y2H	At5g05440 RCAR8	At5g05440-Bam-r	gactcGGATCCTTATTGCCGGTTGGTACTTCGAGC
445	Y2H	RCAR 2	At4g01026_SalI_r	gatcgtcgacCGAAGGTTGGTTTCTGTATGATTCTTCTC
446	Y2H	RCAR 2	At4g01026_EcoRI	gatcgaattcAtggagatgatcggaggagacgatac
451	Y2H	RCAR9	At2g40330_SalI_r	gatcgtcgacCGCGAGAATTTAGAAGTGTTCTCGGCG
452	Y2H	RCAR9	At2g40330_ecoRI	gatcgaattcAtgccaacgtcgatacagtttcagag
456	Y2H	RCAR10	At2g38310_sal1_r	gatcgtcgacCGCAGAGACATCTTCTTCTTGCTCTC
457	Y2H	RCAR10	At2g38310_ecoRI_f	gatcgaattcatgcttgccgttcaccgtccttcttcc
468	Y2H	At5g45860	RCAR5 BamH1r	gatcggatccTTACAACTTTAGATGAGCCACCCTCTCCG
469	Y2H	At5g45860	RCAR5 EcoR1f	gatcgaattcatggaaacttctcaaaaatatcatacgtgcgg
586	Y2H	PP2C At5g51760	5g51760oS_Bamfor	tataggatccactgaaatctacagaacaa
587	Y2H	PP2C At5g51760	5g51760oT_Pstrev	tatactgcagctgagagctattcttgagatc
590	Y2H	RCAR4	RCAR4oS_Ecofor	tatagaattcaacggtgacgaaacaaagaag
591	Y2H	RCAR4	RCAR4oT_Pstrev	tatactgcagtatcttcttctccatagattc
594	Y2H	RCAR6 At5g45870	RCAR6oS_Ecofor	tatagaattcaaaacatctcaagaacagcatg
595	Y2H	RCAR6 At5g45870	RCAR6oT_Pstrev	tatactgcagagtgagctccatcatcttctc
598	Y2H	RCAR7 At4g18620	RCAR7oS_Ecofor	tatagaattcgaaagttctaagcaaaaacgatg
599	Y2H	RCAR7 At4g18620	RCAR7oT_Pstrev	tatactgcagcttcatcattttctttgtgagc
602	Y2H	RCAR13	RCAR13oS_Ecofor	tatagaattcaatcttgctccaatccatgatc
603	Y2H	RCAR13	RCAR13oT_Pstrev	tatactgcagggtcggagaagccgtggaaatg
606	Y2H	RCAR14	RCAR14oS_Ecofor	tatagaattcagctcatccccggccgtgaa
607	Y2H	RCAR14	RCAR14oT_Pstrev	tatactgcagttcatcatcatgcataggtgc
623	PP	At5g45870	RCAR6pps_for	TTGTAGGATCCATGAAAACATCTCAAGAAC
624	PP	At5g45871	RCAR6pps_rev	TTGTACTTAAGTTAAGTGAGCTCCATC
638	Y2H	At1g07430 cDNA	At1g07430 EcoRI	gatcgaattcATGGCGGATATTTGTTATGAAGACG
639	Y2H	At1g47380 cDNA	At1g47380 EcoRI	gatcgaattcATGTCAACAAAGGGAGAACATCATAC
640	Y2H	At1g47380 cDNA	At1g47380 BamHI	gatcggatccGTTGCTTTGCCAATAATTTGC
641	Y2H	At2g29380 cDNA	At2g29380 SmaI	gatccccgggATGGCCGAGATATGTTACGAAG
642	Y2H	At2g29380 cDNA	At2g29380 PstI rev	gatcctgcagTTATCTTCTGAGATCAATCACAACG
643	Y2H	At3g11410 cDNA	At3g11410 SmaI	gatccccgggATGGCTGGGATTTGTTGCG
644	Y2H	At3g11410 cDNA	At3g11410 PstI rev	gatcctgcagCTAATACTACTACTAATTATAACAC
645	Y2H	At5g02760 cDNA	At5g02760 EcoRI	gatcgaattcATGGTTAAACCCTGTTGGAG
646	Y2H	At5g02760 cDNA	At5g02760 SalI rev	gatcgtcgacTCATGATGTTGAATGCATCGG
647	Y2H	At5g59220 cDNA	At5g59220 PstI rev	gatcctgcagCTACGTGTCTCGTCGTAGATCAAC
797	Y2H	cDNA ABRC	U21376 forY	cga tcg aat tcg cga cgt cag gta c
798	Y2H	cDNA ABRC	U21376 revY	cga tcg gat cct taa gcc ttg ctc
799	Y2H	cDNA ABRC	U61154 forY	cga tcg aat tcg ctg gtg tcc aga c
800	Y2H	cDNA ABRC	U61154 revY	cga tcg gat cct cat gat tca gac
801	Y2H	cDNA ABRC	U13643 forY	cga tcg aat tcg ggt tga gtg gtg
802	Y2H	cDNA ABRC	U13643 revY	cga tcg gat cct tag gca cta gtt tg
803	Y2H	cDNA ABRC	U20592 forY	cga tcg aat tcg tag agg cag agg
804	Y2H	cDNA ABRC	U20592 revY	cga tcg gat ccc taa ccc tca gac
805	Y2H	cDNA ABRC	U11943 forY	cga tcg aat tcg ccg acg tag caa c
806	Y2H	cDNA ABRC	U11943 revY	cga tcg gat ccc tat tcc tcg gcc
807	Y2H	cDNA ABRC	U12646 forY	cga tcg aat tcg cga cat cgg gaa c
808	Y2H	cDNA ABRC	U12646 revY	cga tcg gat cct taa gct ttg ttg acg
920	Y2H	At5g27930 cDNA	5g27930 EcoRI f	gatcgaattcGGACATTTCTCATCGATGTTC
921	Y2H	At5g27930 cDNA	5g27930 BamHI r	gatcggatccTTACTTTAAAATCGTCATGG
922	Y2H	At4g11040 cDNA	4g11040 EcoRI f	gatccccgggaGCTGGGATTTGTTGCGG
923	Y2H	At4g11040 cDNA	4g11040 BamHI r	gatcctgcagCTAATACTACTACTAATTATAACAC
924	Y2H	At2g40180 PP2C5	2g40180 SmaI f	gatccccgggaGAGAGTGCGATAAGAGAAGG
925	Y2H	At2g40180 PP2C5	2g40180 PstI r	gatcctgcagAAGTTTAGCTAATGTACTATGTG
926	Y2H	At1g07630 PLL5	1g07630 EcoRI f	gatcgaattcGGTAACGGAGTAACAAAACTG
927	Y2H	At1g07630 PLL5	1g07630 SalI r	gatcgtcgacCTATATTTGTGCTGTGATCAC
928	Y2H	At5g66720 cDNA	5g66720 EcoRI f	gatcgaattcTCAGCGACTGCTCTCTCGAG
929	Y2H	At5g66720 cDNA	5g66720 BamHI r	gatcggatccGGTATCGTCAGATTTTGGCG
930	Y2H	At1g48040 cDNA	1g48040 SmaI f	gatccccgggaGTTGCTGAAGCTGAGATC
931	Y2H	At1g48040 cDNA	1g48040 PstI	gatcctgcagTCACTCGCCTGCAAGC
932	Y2H	At5g66080 cDNA	5g66080 EcoRI f	gatcgaattcTTATCCCTTTTCTTCAACTT
933	Y2H	At5g66080 cDNA	5g66080 BamHI	gatcggatccGAAGAGTGAACCCAAAAAG
934	Y2H	At2g20050 cDNA	2g20050 SmaI f	gatccccgggaAAGAACGTAATAAAGCCCTC
935	Y2H	At2g20050 cDNA	2g20050 PstI r	gatcctgcagTTACCATTCTTCAAGCCAG

936	Y2H	At1g07160 cDNA	1g07160 SmaI f	gatccccgggaTCGTCTTCAGTTGCCGTTTG
937	Y2H	At1g07160 cDNA	1g07160 PstI r	gatcctgcagTTGTTATTCTGTCTCTACATTTC
938	Y2H	At2g33700 cDNA	2g33700 EcoRI f	gatcgaattcAGTATGGATTTTTCACCTTTG
939	Y2H	At2g33700 cDNA	2g33700 SalI r	gatcgtcgacGTCTCACAAAGTAACGTACAAC
940	Y2H	At1g79630 cDNA	1g79630 EcoRI f	gatcgaattcGGTCTGTGTTATTCAGTAGATAG
941	Y2H	At1g79630 cDNA	1g79630 BamHI r	gatcggatccAAGTCACAACCATTTTCTCC
942	Y2H	At4g31750 cDNA	4g31750 SmaI f	gatccccgggaGGATATCTGAATTCTGTTTTG
943	Y2H	At4g31750 cDNA	4g31750 PstI r	gatcctgcagAAACCCGAAAAGGATGTTAC
944	Y2H	At3g16560 cDNA	3g16560 EcoRI f	gatcgaattcCAGGAAGGGACTGATCCGTA
945	Y2H	At3g16560 cDNA	3g16560 SalI r	gatcgtcgacTCACACGAACGTAGAAGCT
958	PP	RCAR7	R7 pSK f	gatcggatcctgatggaaagttc
959	PP	RCAR7	R7 pSK r	gcccgagctcttacttcatcatt
1060	Y2H	At5g46790	RCAR12 Pst r	gactct gcagatcattacctaacctgagaag
1061	Y2H	At1g01360	RCAR1 Pst rev	gactctgcagtcactgagtaatgtcctgagaagc
1062	Y2H	At5g46790	5g46790 BamHI f	gactggatccgtatggcgaattcagagtcc
1063	Y2H	At2g29380	2g29380 Sma f	gatccccgggagccgagatatgttacgaagtag
1064	Y2H	At2g29380	#4 SmaI fwd STA	gactcccggggatggccgagatatgttacg
1065	Y2H	At3g11410	#1 SmaI fwd STA	gactcccggggatggctgggatttgttgc
1066	Y2H	At3g11410	3g11410 SmaI f	gatccccgggagctgggatttgttgcgg
1067	Y2H	At5g53160	RCAR3 PstI rev	gactctgcagttagactctcgattctgtcgtg
1068	Y2H	At1g16220	16220 BamHI rev	gactggatcctcacaaccattttctccagc
1069	Y2H	At1g16220	16220 EcoRI fwd	gactgaattcatgggtttgtgtcattcg
1075	Y2H	MLP34	MLP 34 fw (EcoRI)	ttgtagaattcatggcaaaaactgaagc
1076	Y2H	MLP34	MLP 34 rev (SalI)	ttgtagtcgacctattcctcggccaagag
1131	Y2H	SnRK2.3	SnRK2.3 NotI f	gactgcggccgccATGGATCGAGCTCCGG
1132	Y2H	SnRK2.3	SnRK2.3 BglII r	gactagatctttagagagcgtaaactatctc
1133	Y2H	SnRK2.6	SnRK2.6 Not f	gactgcggccgccATGGATCGACCAGCAGTG
1134	Y2H	SnRK2.6	SnRK2.6 BamHI r	gactggatcctcacattgcgtacacaatc
1143	Y3H	WIN2	WIN2 BamHI r	gactggatccaaacccgaaaaggatgttac
1144	Y3H	WIN2	WIN2 NotI fwd	gatgcggccgccATGGGATATCTGAATTCTG
1145	Y3H	RCAR1	RCAR1 NotI fwd	gactgcggccgccATGATGGACGGCGTTGA
1146	Y3H	RCAR1	RCAR1 BglII rev	gactagatcttcactgagtaatgtcctg
1147	Y3H	RCAR9	RCAR9 NotI fwd	gactgcggccgccATGCCAACGTCGATACAG
1148	Y3H	RCAR11	RCAR11 NotI fwd	gactgcggccgccATGCCTTCGGAGTTAACAC
1155	PP	5g27930	5g27930 EcoRI f	gatcgaattcATGGGACATTTCTCATCG
1156	PP	1g79630	1g79630 EcoRI f	gatcgaattcATGGGTCTGTGTTATTCAG
1209	Y2H	HopW1-1	HOPW1fl_GADfor	GCACGGAATTCATGAGTCCAGCTCAGATT
1210	Y2H	HopW1-1	HOPW1truGADfor	GCACGGAATTCATGACGCGTGTTATCAAAGGG
1211	Y2H	HopW1-1	HOPW1_GADrev	GCAGTCGACTCAACGGTCTTTGGAGGA
1212	Expr	HopW1-1	HOPW1fl_QEfor	GCGCATGCACATGAGTCCAGCTCAGATT
1213	Expr	HopW1-1	HOPW1truQEfor	GCGCATGCACATGACGCGTGTTATCAAAGGG
1214	Expr	HopW1-1	HOPW1_QErev	GCAGGATCCACGGTCTTTGGAGGACTT
1215	SDM	>AT2G26980	CIPK3 SDM f	GATGGACTCTTGCATGACTCGTGTGGAACACC
1216	SDM	>AT2G26980	CIPK3 SDM r	GGTGTTCCACACGAGTCATGCAAGAGTCCATC
1217	SDM	>AT5G10930	CIPK5 SDM f	GGATTGCTTCATGACCAGTGTGGAACTCCGG
1218	SDM	>AT5G10930	CIPK5 SDM r	CCGGAGTTCCACACTGGTCATGAAGCAATCC
1219	SDM	>AT4G24400	CIPK8 SDM f	GTTACCATCCTAAAGGACACATGTGGAACTCCC
1220	SDM	>AT4G24400	CIPK8 SDM r	GGGAGTTCCACATGTGTCCTTTAGGATGGTAAC
1221	SDM	>AT5G07070	CIPK2 SDM f	GGTCTTCTACATGACACTTGTGGTACACCTGC
1222	SDM	>AT5G07070	CIPK2 SDM r	GCAGGTGTACCACAAGTGTCATGTAGAAGACC
1223	SDM	>AT1G01140	CIPK9 SDM f	GGTTTGCTTCATGACGCTTGTGGAACGCCAAAC
1224	SDM	>AT1G01140	CIPK9 SDM r	GTTTGGCGTTCCACAAGCGTCATGAAGCAAACC
1225	SDM	>AT2G30360	CIPK11 SDM f	GGGTTGTTACATGACTTGTGTGGTACTCCGG
1226	SDM	>AT2G30360	CIPK11 SDM r	CCGGAGTACCACACAAGTCATGTAACAACCC
1227	SDM	>AT5G01820	CIPK14 SDM f	GCCTCCTCCGACCACTCTGTGGTACGCCG
1228	SDM	>AT5G01820	CIPK14 SDM r	CGGCGTACCACAGAGTGGTCGGAGGAGGC
1229	SDM	>AT5G01810	CIPK15 SDM f	CGGGTTGCTGCATGACACATGCGGGACCC
1230	SDM	>AT5G01810	CIPK15 SDM r	GGGTCCCGCATGTGTCATGCAGCAACCCG
1231	SDM	>AT5G57630	CIPK21 SDM f	CGGGGGATATGCTCTCTGACGCTTGTGGC
1232	SDM	>AT5G57630	CIPK21 SDM r	GCCACAAGCGTCAGAGAGCATATCCCCCG
1233	SDM	>AT5G35410	CIPK24 SDM f	CTTCTGCGTGACACATGTGGAACTCCGAAC
1234	SDM	>AT5G35410	CIPK24 SDM r	GTTCGGAGTTCCACATGTGTCACGCAGAAG

1235	SDM	>AT4G18700	CIPK12 SDM f	GGGCTTTTTCATGACTTTTGTGGTACTCCTGC
1236	SDM	>AT4G18700	CIPK12 SDM r	GCAGGAGTACCACAAAAGTCATGAAAAAGCCC
1239	SDM	>AT2G34180	CIPK13 SDM f	GGAATCTGTCAAGACTTTTGCGGGACACCAG
1240	SDM	>AT2G34180	CIPK13 SDM r	CTGGTGTCCCGCAAAAGTCTTGACAGATTCC
1241	SDM	>AT1G29230	CIPK18 SDM f	CGGGCTCTGCCACGACTTTTGCGGGACTC
1242	SDM	>AT1G29230	CIPK18 SDM r	GAGTCCCGCAAAAGTCGTGGCAGAGCCCG
1243	SDM	>AT5G45810	CIPK19 SDM f	GGGTTATTTCATGACTTTTGTGGGACCCCTGC
1244	SDM	>AT5G45810	CIPK19 SDM r	GCAGGGGTCCCACAAAAGTCATGAAATAACCC
1245	SDM	>AT5G45820	CIPK20 SDM f	GGCTTGCTTCACGACACATGTGGAACACCTG
1246	SDM	>AT5G45820	CIPK20 SDM r	CAGGTGTTCCACATGTGTCGTGAAGCAAGCC
1247	SDM	>AT2G25090	CIPK16 SDM f	TACATGACCGGTGCGGGACACCGGCTTACG
1248	SDM	>AT2G25090	CIPK16 SDM r	CACCGGTCATGTAAAAGATCATCGCTGCTAC
1348	Y2H	ABI5	abr5Gfor	ttgtaggatccatatggtaactagagaaacg
1349	Y2H	ABI5	abr5Grev	ttgtagtcgacttagagtggacaactcg
1382	PP	pSASFa MLPI	MLPI EcoRI fwd	GACTGAATTCATGACAATGAAAATGAGTGG
1383	PP	pSASFa MLPI	MLPI PstI rev	GACTCTGCAGTTATTCGTGAAATTTAAGAAG
1384	PP	pSASFa MLPH	MLPH PstI rev	GACTCTGCAGCTATTCCTCGGATAAGAG
1385	PP	pSASFa MLPH	MLPH EcoRI fwd	GACTGAATTCATGGCAGAGACTACAAAG
1391	PP	pSASFa MLPG	MLPG EcoRI fwd	GACTGAATTCATGGCGACGTCAGGAAC
1392	PP	pSASFa MLPG	MLPG PstI rev	GACTCTGCAGTTAGGCTTTGAGAACGTG
1393	PP	pSK pPR1::LUC	pPR1_SacII fwd	TCTCCGCGGCACAAAAGTAGATCGGTCAC
1394	PP	pSK pPR1::LUC	pPR1_BamHI rev	TCTGGATCCATGATTTTGGGGTTCGTAAAC
1395	PP	pSASFa MLP31	MLP31 EcoRI fwd	GACTGAATTCATGGCGACTAAAATGGCC
1396	PP	pSASFa MLP31	MLP31 PstI rev	GACTCTGCAGCTATTCCTCGTTCAGGAG
1397	PP	pSASFa MLPA	MLPA EcoRI fwd	GACTGAATTCATGGCGACGTCAGGTAC
1398	PP	pSASFa MLPC	MLPC EcoRI fwd	GACTGAATTCATGGGGTTGAGTGGTG
1399	PP	pSASFa MLPD	MLPD EcoRI fwd	GACTGAATTCATGGTAGAGGCAGAGG
1400	PP	pSASFa MLPF	MLPF EcoRI fwd	GACTGAATTCATGGCGACATCGGGAAC
1476	PP	pSASFa PP2C6	PP2C6 EcoRI fw	GACTGGAATTCATGCCCAAGATCTGCTGCTCTC
1477	PP	pSASFa MLP B	MLP_B EcoRI fw	GACTGGAATTCATGGCTGGTGTCCAGAC
1478	Expr	pQE70 WIN2	WIN2 BamHI rv	GACTGGATCCGGTTGATGAGTCACCG
1479	Expr	pQE70 WIN2	WIN2 SphI fw	GACTGCATGCGGATGGGATATCTGAATTCTGT
1480	PP	pSASFa WIN2	WIN2 XmaI fw	gactCCCGGGATGGGATATCTGAATTCTGTTTTGTC
1492	PP	pPR1::LUC	pPR1 SacII L fwd	tgtccgcggcgtaataatatg?tatggtgtc
1493	SDM	WIN2	WIN2 G72D fwd	gtctttgatggacatgacggtgcacgtgcagctg
1494	SDM	WIN2	WIN2 W183A fwd	ggaggatttgtcatggcggctggaacatggagag
1496	Y2H	AHG1 no ATG	AHG1 BamHI f	gactggattcgaacaatttcaaccggacgtg
1497	Y3H	SnRK2.2	SnRK2.2 NotI f	gactgcggccgccatggatccggcgactaa
1498	Y3H	SnRK2.2	SnRK2.2_neu_BgI	ttgtaagatcttgatggatccggcgactaa
1499	Y2H	SnRK2.2	SnRK2.2_SacI_re	ttgtagagctctcagagagcataaac
1500	Y2H	SnRK2.3	SnRK2.3_SacI_re	ttgtagagctcttagagagcgtaaacta
1501	Y2H	SnRK2.6	Ost1_SalI_rev	ttgtagtcgactcacattgcgtacac
1502	Y2H	SnRK2.6	OST1_neu_BamHI_	ttgtaggatcctgatggatcgaccagcag
1503	PP	HAB2	HAB2 EcoRI f	gactgaattcatggaagagatttcacctg

Lebenslauf

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2002 - 2008	Diplomstudiengang Biologie Leopold-Franzens-Universität Innsbruck, Österreich einschließlich der Diplomarbeit "Apoptose-Induktion durch pflanzliche Wirkstoffe in Leukämie- und Neuroblastomzellen", angefertigt im Molekularbiologischen Forschungslabor, Department für Pädiatrie, Medizinische Universität Innsbruck, Österreich
06/2008	Abschluss als Magister der Naturwissenschaften (Mag. rer. nat.)
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Publikationen

Ausserlechner, M. J., Hagenbuchner, J., Fuchs, S., Geiger, K., Obexer, P. (2012), FOXO Transcription Factors as Potential Therapeutic Targets in Neuroblastoma. Neuroblastoma – Present and Future, Prof. Hiroyuki Shimada (Ed.), ISBN: 978-953-307-016-2, InTech.

Fuchs, S., Grill, E., Meskiene, I. and Schweighofer, A. (2012), Type 2C protein phosphatases in plants. FEBS Journal, 280: 681–693. doi: 10.1111/j.1742-4658.2012.08670.x

Fuchs, S., Tischer, S., Wunschel, C., Grill, E. (2013), RCAR7 is a functional ABA receptor in *Arabidopsis thaliana*. (in preparation)

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Anlage 5

Eidesstattliche Erklärung

Ich erkläre an Eides statt, dass ich die bei der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt (promotionsführende Einrichtung) der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:

Protein Phosphatases 2C of Arabidopsis thaliana in Stress Signaling

in am Lehrstuhl für Botanik

(Fakultät, Institut, Lehrstuhl, Klinik, Krankenhaus, Abteilung)

unter der Anleitung und Betreuung durch: Prof. Dr. Erwin Grill

ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 6 Abs. 6 und 7 Satz 2 angegebenen Hilfsmittel benutzt habe.

☑ Ich habe keine Organisation eingeschaltet, die gegen Entgelt Betreuerinnen und Betreuer für die Anfertigung von Dissertationen sucht, oder die mir obliegenden Pflichten hinsichtlich der Prüfungsleistungen für mich ganz oder teilweise erledigt.

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☑ Ich habe den angestrebten Doktorgrad noch nicht erworben und bin nicht in einem früheren Promotionsverfahren für den angestrebten Doktorgrad endgültig gescheitert.

☐ Ich habe bereits am .....

bei der Fakultät für .....

der Hochschule .....

unter Vorlage einer Dissertation mit dem Thema .....

die Zulassung zur Promotion beantragt mit dem Ergebnis:

.....

Die öffentlich zugängliche Promotionsordnung der TUM ist mir bekannt, insbesondere habe ich die Bedeutung von § 28 (Nichtigkeit der Promotion) und § 29 (Entzug des Doktorgrades) zur Kenntnis genommen. Ich bin mir der Konsequenzen einer falschen Eidesstattlichen Erklärung bewusst.

Mit der Aufnahme meiner personenbezogenen Daten in die Alumni-Datei bei der TUM bin ich

☑ einverstanden ☐ nicht einverstanden.

Freising, 06.06.2013, Stef Jeuos

(Ort, Datum, Unterschrift)