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TIIVISTELMÄ

Selviytyäkseen ja kasvaakseen luonnonolosuhteissa kasvien täytyy mukautua elinkaarensa aikana kohtaamiinsa ympäristöolosuhteisiin. Kasvu- ja stressivasteet on tasapainotettava tarkasti vajavaisen kasvun ja kuoleman välttämiseksi. Viestinvälitysverkosto, jonka kautta kasvit reagoivat stressitekijöihin, on laaja ja sisältää useita säätelytasoja.

Proteiinikinaasit, jotka katalysoivat proteiinien toimintaa muokkaavaa fosforylaatiota, ovat tärkeä osa stressitilojen aikaista signaalinvälitystä. SnRK-kinaasiperheen (SNF1-related protein kinase) jäsenet ovat keskeisiä kasvien stressivasteita ja energiatasapainoa sääteleviä tekijöitä. Väitöskirjatyössäni tutkittiin näiden kinaasien säätelymekanismeja ja sitä, miten ne osallistuvat erilaisiin signaalinvälitysketjuihin. Tutkimuksessa selvitettiin, miten kinaasien omat alayksiköt tai muut proteiinit säätelevät SnRK-geeniperheen jäsenten toimintaa erilaisissa olosuhteissa, miten kinaasien toimintaa voitaisiin hallita kemikaalien avulla, ja onko niillä vaikutusta kasvien pintasolukkoa peittävän ja veden haihtumista estävän kutikulan muodostumiseen.

Kinaasien toimintaa tutkittiin määrittämällä niiden aktiivisuustasoja, mittaamalla kinaasien ilmenemistä eri olosuhteissa, mukaanlukien kuivuus- ja suolastressin aikana, tarkastelemalla signaalinvälityksen mutanttien käyttäytymistä, ja mallintamalla kinaasien vuorovaikutusta muiden molekyylien kanssa. *In vitro*-määritykset osoittivat, että KING (KIN gamma) –proteiinit säätelevät SnRK2-proteiiniperheen jäsenten aktiivisuutta. KING-proteiinien on jo aiemmin todettu mahdollisesti säätelevän kasvien abskissihappo-välitteistä signalointireittiä. Abskissihappo (ABA) on kasvihormoni joka säätelee kasvin kehitystä ja stressivasteita. KING-proteiinien havaittiin olevan todennäköisiä negatiivisia ABA-signaalinvälityksen säätelijöitä myös *in vivo*, joskin niiden vaikutus SnRK2-proteiineihin jäi vielä epäselväksi. ABA:sta riippuvan kutikulan muodostumisen säätelyreitin todettiin erkaantuvan pääasiallisesta ABA:n signaalinvälitysketjusta. Pladienolide B –kemikaalin todettiin pystyvän säätelemään SnRK2.6-proteiinin aktiivisuutta. Tätä kemikaalien aiheuttamaa aktiivisuuden säätelyä voitaisiin hyödyntää SnRK2-proteiinien toimintojen tutkimisessa tai kasvien stressinsiedon parantamisessa. SnRK2-proteiinien tutkimisen lisäksi väitöskirjassa tarkasteltiin muiden SnRK-kinaasiperheiden jäsenten toimintaa sääteleviä tekijöitä. Osoittautui, että Gemnivirus REP-Interacting Kinase (GRIK) –kinaasit, joiden on aiemmin tiedetty vaikuttavan SnRK1-kinaasien toimintaan, osallistuvat suolansietokyvyn säätelyyn ja kykenevät fosforyloimaan yhtä SnRK3-kinaasiperheen jäsentä *in vitro*.

Väitöskirjatutkimukseni selkeytti SnRK-proteiinien toimintaa ja säätelyä sekä toi lisätietoa niiden osallistumisesta kasvien stressivasteiden säätelyyn. Tietoa stressivasteiden muodostumisesta ja säätelystä voidaan hyödyntää esim. stressiä sietävien viljakasvien jalostuksessa.

ASIASANAT: ympäristöstressi, abskissihappo, proteiinikinaasi, SnRK, kutikula

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ABSTRACT

Plants regulate their responses to changing environmental conditions to cope with stresses and survive under natural growth habitats. Growth and stress responses must be strictly balanced to prevent poor survival and compromised growth. The network of stress responses to various biotic and abiotic stressors is vast, interconnected, and includes multiple levels of regulation.

Protein kinases play important roles in stress signalling as catalysts of phosphorylation, a modification that allows for a convenient way of rapidly adjusting protein functions. SNF1-RELATED PROTEIN KINASEs (SnRKs) are a central kinase superfamily that is involved in stress responses and energy balance in plants. The aim of my doctoral thesis was to investigate the roles of SnRKs in various signalling pathways. This included information about how SnRKs are regulated by upstream elements and potential subunits, how their activities could be manipulated chemically, and whether they are involved in regulation of cuticle formation.

SnRKs were studied by measuring their kinase activities, by analysing the expression levels in various conditions, including drought and salt stress, by characterisation of several signalling mutants, and by modelling of the interaction between SnRK2.6 and other molecules. The activities of SnRK2s were influenced *in vitro* by KIN GAMMA (KING) proteins, which had been previously identified as putative regulators of signalling related to abscisic acid (ABA). ABA is a plant hormone that is involved in plant development and stress responses. My studies suggested that KINGs are probable negative regulators of ABA signalling also *in vivo*, although their effects on SnRK2s remained less clear. A potential activator of ABA signalling, pladienolide B, activated SnRK2.6. This information is useful for further studies of the functions of SnRK2s, or for enhancing stress tolerance of plants. In addition to the research on regulation of SnRK2s, their downstream signalling pathways were investigated in regards to cuticle formation. The ABA-dependent regulation pathway of cuticle formation was found to deviate from the main ABA signalling pathway at SnRK2s. In addition to SnRK2s, regulators of other members of the SnRK superfamily were also examined. GEMINIVIRUS REP-INDUCING KINASEs (GRIKs), previously identified as upstream regulators of SnRK1s, showed a capacity to phosphorylate a member of the third SnRK family (SnRK3s) *in vitro*. *In vivo* experiments also showed that GRIKS are involved in NaCl tolerance.

The results of this thesis clarified several functions of SnRKs in plant stress signalling. The knowledge of the induction and regulation of stress responses in plants is valuable for the breeding of economically important plants, e.g. stress-tolerant crops.

KEYWORDS: abiotic stress, abscisic acid, protein kinases, SNF1-related protein kinases, cuticle, pladienolide B

Abbreviations

5PTase13	TYPE I INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 13
ABA	Abscisic acid
ABRE	ABA-responsive element
AHA1	ARABIDOPSIS H^+-ATPASE 1
AHK	ARABIDOPSIS HISTIDINE KINASE
AKS1	ABA-RESPONSIVE KINASE SUBSTRATE 1
AKT	ARABIDOPSIS K^+ TRANSPORTER
AMPK	AMP-ACTIVATED PROTEIN KINASE
AREB	ABA-RESPONSIVE ELEMENT BINDING
ATHB	HOMEOBOX-LEUCINE ZIPPER PROTEIN ATHB
ATP	Adenosine triphosphate
BAK1	BRASSINOSTEROID INSENSITIVE 1 -ASSOCIATED RECEPTOR KINASE 1
BIN2	BRASSINOSTEROID INSENSITIVE 2
BRM	SWI/SNF CHROMATIN-REMODELING ATPASE BRAHMA
CaM	Calmodulin
CaMK	CALMODULIN-BINDING KINASE
CARK1	CYTOSOLIC ABA RECEPTOR KINASE 1
CAT	CATALASE
CAX	VACUOLAR CATION/PROTON EXCHANGER
CBL	CALCINEURIN-B-LIKE
CDPK	Ca^{2+}-DEPENDENT PROTEIN KINASE
CDL1	CDG1-LIKE 1
CIPK	CBL-INTERACTING PROTEIN KINASE
CK2	CASEIN KINASE 2
CLC	CHLORIDE CHANNEL
COR	COLD-REGULATED
CRE1	CYTOKININ RESPONSE 1
CRL4	CULLIN4-RING E3 LIGASE

DMSO	Dimethylsulfoxide
EIN3	ETHYLENE INSENSITIVE 3
ER	Endoplastic reticulum
FLZ	FCS-LIKE ZINC FINGER
GORK	GATED OUTWARD-RECTIFYING K^+ CHANNEL
GRIK	GEMINIVIRUS REP-INTERACTING KINASE
HKT	HIGH-AFFINITY K^+ TRANSPORTER
HMGR	3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE
IPTG	Isopropyl-beta-D-thiogalactopyranoside
KAT	K^+ CHANNEL IN ARABIDOPSIS THALIANA
KEG	E3 UBIQUITIN-PROTEIN LIGASE KEG
KING	KIN GAMMA
KUP	K^+ UPTAKE TRANSPORTER
LEA	LATE EMBRYOGENESIS ABUNDANT
MAPK	MITOGEN-ACTIVATED PROTEIN KINASE
NDPK2	NUCLEOSIDE DIPHOSPHATE KINASE II, CHLOROPLASTIC
NHX	Na^+/H^+ EXCHANGER
NMT	GLYCYLPEPTIDE N-TETRADECANOYLTRANSFERASE
NO	Nitric oxide
NR	NITRITE REDUCTASE
NRT	HIGH-AFFINITY NITRATE TRANSPORTER
OST1	OPEN STOMATA 1
PIP1;2	AQUAPORIN PIP1-2
PKS	PROTEIN KINASE S
PYR1/PYL/	PYRABACTIN RESISTANCE 1/ PYR1-LIKE/ REGULATORY
RCAR	COMPONENTS OF ABA RECEPTORS
PP2-B11	F-BOX PROTEIN PP2-B11
PP2C	PROTEIN PHOSPHATASE 2C
PRL1	PROTEIN PLEIOTROPIC REGULATORY LOCUS 1
PUB	E3 UBIQUITIN-PROTEIN LIGASE PUB
QUAC1	QUICK ANION CHANNEL ASSOCIATED 1
RAV1	AP2/ERF AND B3 DOMAIN-CONTAINING TRANSCRIPTION FACTOR RAV1
RBOH	RESPIRATORY BURST OXIDASE HOMOLOGUE
RGLG	E3 UBIQUITIN-PROTEIN LIGASE RGLG
RIFP1	RCAR3 INTERACTING F-BOX PROTEIN 1

ROS	Reactive oxygen species
RSL1	RING FINGER OF SEED LONGEVITY1
SCS	SnRK2-INTERACTING CALCIUM SENSOR
SIZ1	E3 SUMO-PROTEIN LIGASE SIZ1
SLAC1	SLOW ANION CHANNEL 1
SLAH3	SLAC1-HOMOLOG PROTEIN 3
SnAK	SNRK1-ACTIVATING KINASE
SNF1	SUCROSE NON-FERMENTING 1
SNF4	SUCROSE NON-FERMENTING 4
SnRK	SNF1-RELATED KINASE
SOS	SALT OVERSENSITIVE
SPS	SUCROSE PHOSPHATE SYNTHASE
SR45	SERINE/ARGININE-RICH SPLICING FACTOR SR45
T6P	TREHALOSE-6-PHOSPHATASE
TB	Toluidine blue
TOPP1	TYPE ONE PROTEIN PHOSPHATASE 1
TOR	TARGET OF RAPAMYCIN
V-ATPase	V-TYPE PROTON ATPASE

List of Original Publications

This thesis is based on the following scientific articles, referred to in the text by their Roman numerals:

I Punkkinen M, Dessineouk K, Fujii H (2019) Arabidopsis KIN Gamma subunit 1 (KING1) has potential to regulate activity of SUCROSE NONFERMENTING 1-RELATED PROTEIN KINASE 2s (SnRK2s) in vitro. Biologia Plantarum 63: 54-58

II Punkkinen M, Barajas-Lopez J, Fujii H (2019) Phenotypic analysis of Arabidopsis KIN GAMMA mutants 1 and 2. Manuscript.

III Punkkinen M, Dessineouk K, Mahfouz M, Fujii H (2019) Pladienolide B can increase activity and expression of SnRK2.6. Manuscript.

IV Cui F, Brosché M, Lehtonen M, Amiryousefi A, Xu E, Punkkinen M, Valkonen J, Fujii H, Overmyer K (2016) Dissecting Abscisic acid signaling pathways involved in cuticle formation. Molecular Plant 9: 926-938

V Barajas-Lopez J, Moreno J, Gamez-Arjona F, Pardo J, Punkkinen M, Zhu J-K, Quintero F, Fujii H (2018) Upstream kinases of plant SnRKs are involved in salt stress tolerance. Plant Journal 93: 107-118

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1 Introduction

Throughout the entirety of their lives, plants must adapt to and endure various stress conditions, or perish. These stress conditions can arise from biotic stress factors, including microbes and herbivores, and abiotic stress factors, i.e. environmental conditions that reduce plant growth and yield below optimum levels (Cramer et al. 2011). Environmental stress is a major cause of crop loss, limiting production by up to 70 % (Boyer 1982; Ashraf et al. 2009). By varying estimates only 3.5-10 % of arable land is non-stressed, and both the area of land affected by environmental stress and the severity of the stress are increasing (Velthuizen et al. 2007; Sharma & Lavanya 2002). This is caused by multiple factors, including extreme weather conditions resulting from climate change, and increase in salt water irrigation. As global population grows and arable land becomes less productive, improved crop yields and efficient land use are required for sustained food production (Zhao et al. 2017). An important way of obtaining sufficient crop yields lies in improving plants' tolerance to stress, allowing them to be grown in poorer land and more extreme conditions, with reduced probability of plant death. This requires knowledge of how plants react to stress: the sensors that detect what type of stress is occurring, the signalling cascades that relay the information about the stress to downstream targets, and the responses through which plants react to the stress.

Abiotic stress can be caused by multiple sources, including intense light, toxic metals or ions, mineral deficiency, extreme temperatures, anoxia from submersion, ozone, UV-B irradiation, and/or decreased availability of water due to high ionic concentrations or drying of the soil. Plants also commonly experience a combination of multiple stresses, e.g. heat causes drought, exposing plants simultaneously to heat stress and drought stress, and responses to a combination of stresses can be different than to the single stresses separately (Mittler 2006). It is therefore not surprising that molecular responses to stresses involve crosstalk with multiple signalling pathways, or that the regulation networks that integrate the signals for different stresses are complex and precisely controlled (Hey et al. 2010). Responses to stress can be either reversible or irreversible, and depend greatly on both the affected tissues or organs, and the severity and duration of the stress (Tattersall et al. 2007; Pinheiro & Chaves

2011; Skirycz et al. 2011). Many aspects of this regulation are not completely understood, although a few categorical differences have been identified: inhibition of protein synthesis, upregulation of genes related to protein folding and processing, and increased antioxidant activity are generally early responses, while effects on energy metabolites, including sugars and lipids, are more gradual (Cramer et al. 2011; Bechtold 2018). Additionally, expression of photosynthesis-related genes may be upregulated in response to moderate stress in order to support e.g. increased root growth (Des Marais et al. 2012). Negative feedback regulation and strict control of stress responses are essential, since excessively strong reactions to adverse conditions can lead to suppression of growth and metabolism that harms the plant in the long-term (Irigoyen et al. 2014; Zhang et al. 2017).

1.1 Defence responses to abiotic stress

Plants can respond to stresses with multiple strategies, which can be traditionally grouped to three distinct categories: tolerance, escape, and avoidance through adaptation and acclimatization (Abdelrahman et al. 2017). Escape mechanisms, e.g. acceleration of flowering, speed up the life cycle of the plant so that the seeds for the next generation of plants can be produced before severity of the stress becomes critical. Avoidance consists of mechanisms that limit the extent to which plant experiences the extreme conditions, e.g. prevent the accumulation of toxic ions by maximizing their efflux, and tolerance encompasses mechanisms that allow the plants to cope with the stress in their tissues, e.g. induce production of osmoprotectants.

Plants employ both shared and unique responses to different types of stress. In particular, there is overlap in responses to cold, drought, and salt stress, since all of them limit water availability. Water moves according to the water potential, from higher to lower potential energy (Verslues et al. 2006). Cold, drought, and salt stress limit water availability through freezing, reduction in the volume of water in soil, or increased concentrations of ions in soil, respectively. The stress that results from this limited water availability is called osmotic stress. Common responses to osmotic stress include accumulation of osmocompatible solutes, such as proline, glycine betaine, dehydrins, LATE EMBRYOGENESIS ABUNDANT (LEA) proteins, and sugars (glucose, fructose, sucrose, and raffinose). Increased solute concentration decreases water potential in cells, discouraging water escape and generating turgor (Verslues et al. 2006; Kishor et al. 1995; Hayashi et al. 1997). Plants under osmotic stress also change their growth patterns: enhanced root growth expands the area from which they can collect water and nutrients, and limited shoot growth minimises

the area of transpirational surfaces (Munns & Sharp 1993). The changes in growth patterns may also include adaptive senescence, wherein leaves die and are abscised prematurely. This further reduces the area of transpirational surfaces and thus water loss, and allows remobilisation of nutrients, importantly nitrogen, from old tissues to meristems and storage tissues (Zhao et al. 2016). Finally, osmotic stress induces changes in redox buffering and energy metabolism for prevention of damage that could be caused by excessive production of reactive oxygen species (ROS) and for maintenance of redox balance in the cells (Sharma et al. 2011).

In addition to the osmotic stress component, low temperature stress affects membrane fluidity, protein configuration, and enzyme activity. Vacuolar H^+-ATPase is especially sensitive to cold, its inactivation occurring long before cell injuries are visible and activities of other enzymes decrease. Chilling causes acidification of the cytoplasm and alkalization of the vacuoles, possibly by disrupting H^+ pumping (Kawamura 2008; Yoshida et al. 1989). Plants respond to cold stress by decreasing cerebroside content of their plasma membranes, and by accumulating phospholipids and membrane proteins related to membrane repair, protection, and transport. This helps in preserving membrane fluidity and integrity (Uemura & Steponkus 1994; Uemura et al. 2006). Acclimation to cold is associated with several transcription factors, namely C-REPEAT/DEHYDRATION-RESPONSIVE ELEMENT BINDING FACTORs (CBF/DREBs) and INDUCER OF CBF EXPRESSIONs (ICEs) (Lissarre et al. 2010; Chinnusamy et al. 2007). Temperatures below freezing can cause damage to cell membranes, either through physical damage caused by ice crystals or through shrinking and distortion of the plasma membrane. Freezing tolerance requires membrane cryostability, maintenance of which has been suggested to be mediated by lipid composition of cell membrane, compatible solutes, and hydrophilic proteins, such as COLD-REGULATED (COR) proteins (Thalhammer et al. 2014; Sakamoto & Murata 2001; Thomashow 1998).

Aside from the osmotic stress component caused by changes in ionic concentrations, saline stress causes toxicity from overaccumulation of ions. While osmotic stress occurs soon after exposure to salt, ionic stress generally appears later, since the accumulation of ions to toxic levels is gradual. As the most soluble natural salt, NaCl is the most common contaminating salt, and in most plants Na^+ accumulates to toxic levels before Cl^- (Munns & Tester 2008). Thus, plants' responses to Na^+ have been studied thoroughly. Na^+ toxicity arises from competition with K^+, which disrupts enzymatic processes as well as protein synthesis. Plants can prevent Na^+ toxicity by limiting the uptake of Na^+ and its access to tissues. Uptake of Na^+ through roots can be minimised by increased flow back into the soil through Na^+ efflux channels. Harm to photosynthetic tissues can be reduced by retaining the ions

in roots through minimal upload and maximal retrieval at xylem, and by removing the ions from leaves through maximised phloem loading at leaves (Munns et al. 2006). Additionally, ions can be tolerated in tissues by sequestering them into the vacuole (Ismail & Horie 2017). Several proteins have been identified as important in the control of Na^+ transport. These include certain members of the SnRK3/CIPK kinase family, which can regulate the activities of Na^+ transporters, as detailed later in this thesis. Important Na^+ transporters include plasma membrane Na^+/H^+ antiporter SALT OVERSENSITIVE 1 (SOS1), which is involved in Na^+ efflux from cells and possibly sequestration into vacuole, and HIGH-AFFINITY K^+ TRANSPORTERs (HKTs), which mediate influx of Na^+ and K^+ into cells and could aid in sequestering Na^+ into root cells (Horie et al. 2009). Vacuolar Na^+/H^+ EXCHANGERs (NHXs) are important for compartmentalisation of Na^+ into the vacuole (Bassil et al. 2011). They function in concert with vacuolar H^+ pyrophosphatases, which use breakdown of inorganic pyrophosphatase to pump protons into vacuole, thereby establishing the vacuole-apoplasm proton gradient (Gaxiola et al. 2001).

1.1.1 Signalling molecules in abiotic stress

Initiation of the stress signal is in many cases unclear, but should usually start at a sensor that detects a change in external conditions. Osmosensors are as of yet unknown in plants, but in prokaryotes osmosensors are known to be two-component systems that consist of a histidine kinase and a response regulator (Wang et al. 2015). Candidates for two-component systems in plants include ARABIDOPSIS HISTIDINE KINASEs (AHKs) 1, 2, and 3, CYTOKININ RESPONSE 1 (CRE1), and mechanosensitive channels (Reiser et al. 2003; Tran et al. 2007; Veley et al. 2012). Alternatively, turgor changes during water loss can lead to loss of contact between cell wall and membrane, which could be recognised as a sign of osmotic stress. NDR1 and At14a have been suggested to play a role in this type of signal initiation (Knepper et al. 2011; Lu et al. 2012). Similarly, REDUCED HYPEROSMOLALITY-INDUCED Ca^{2+} INCREASEs (OSCAs), Ca^{2+} permeable channels, have been identified as probable sensors that release a Ca^{2+} signal as membrane tension changes during osmotic stress (Yuan et al. 2014; Liu et al. 2018). After the initiation of the signal, plants employ small molecules that relay the signals in signalling cascades to and between various proteins.

Ionic calcium (Ca^{2+}) is a ubiquitous signalling molecule in physiological processes, including stress signalling. Normally Ca^{2+} is stored in various organelles, and its cytosolic concentration is very low (100-200 nM; Clapham 2007). Calcium is

released to cytosol through calcium channels as stimulus-specific calcium signatures and converted to phosphorylation responses by Ca^{2+}-dependent protein kinases. At least three families of these protein kinases are present in plants: Ca^{2+}-DEPENDENT PROTEIN KINASEs (CDPKs), which bind Ca^{+2} with a calmodulin-like domain; CBL-INTERACTING PROTEIN KINASEs (CIPKs, also known as SnRK3s), which interact with Ca^{2+}-binding CALCINEURIN B-LIKE (CBL) proteins; and CALMODULIN-BINDING KINASEs (CaMKs), which interact with Ca^{2+}-binding Calmodulin (CaM) or Calmodulin-like (CML) proteins (Chen et al. 2017). Ca^{2+} is stored back into organelles by P-type Ca^{2+}-ATPases and tonoplast Ca^{2+}/H^{+} exchangers of the CATION EXCHANGER (CAX) family (Tang & Luan 2017).

Reactive oxygen species (ROS) have multiple effects on plants: On one hand, they are oxidising molecules that are produced as a consequence of stress. Uncontrolled production of ROS is liable to cause damage to cells, which plants attempt to prevent by employing redox scavenging mechanisms. On the other hand, they also function as signalling molecules during stress responses (Mittler et al. 2011). Due to these dual functions as both toxins and signalling molecules, production of ROS has to be tightly controlled. A part of pathogen-related signalling and osmotic stress responses is production of superoxide by the NADPH oxidases, RESPIRATORY BURST OXIDASE HOMOLOGUEs (RBOHs) (Joshi-Saha et al. 2011). They are a point of convergence in multiple pathways, since several proteins and signalling molecules that are involved in various pathways regulate their activity. These regulators include kinases and phosphatases of the SnRK2, SnRK3, and PP2C families, and small guanosine triphosphates (Sirichandra et al. 2009; Han et al. 2018; Qu et al. 2017). RBOHF, which activates in response to various stimuli and physiological processes, appears to be the most multifunctional RBOH. It works together with RBOHD during ABA signalling in guard cells, and both of them are required for accumulation of reactive oxygen intermediates in defence responses (Qu et al. 2017; Han et al. 2018; Kwak et al. 2003).

Phytohormones play important roles in stress responses. In particular, abscisic acid (ABA) is well-known for its involvement in multiple stress responses, including osmotic stress. ABA is crucial in control of stomatal movements and several other stress responses, such as accumulation of osmocompatible solutes (Fujita et al. 2009). In addition to its roles in stress responses, ABA is central in many developmental processes, including embryo and seed development, dormancy/germination, seedling establishment, vegetative development, reproduction, and leaf senescence. It functions antagonistically to gibberellins during germination, promoting dormancy (Zhao et al. 2017). ABA is produced primarily in vascular tissues, but its whole-plant-wide effects indicate the presence of an intercellular transportation and

signalling network. Some ABA transporters have been identified, including vascularly expressed ABCG25, guard cell-expressed importer ABCG40, and AIT1/NRT1.2, which mediates cellular ABA uptake likely at the site of biosynthesis. Mutants of these transporters have reduced sensitivity to ABA but they are still capable of responding to it, indicating the presence of as-of-yet unknown transporters (Umezawa et al. 2010; Kanno et al. 2012).

ABA has been investigated as a potential treatment agent for improving crop stress tolerance. However, due to its system-wide effects on plants and quick degradation, ABA derivatives or analogues that have more favourable features have been proposed as alternative treatment agents that could also contribute to research about the ABA signalling mechanisms (Ito et al. 2015). Several of these have been discovered, including ABA agonists pyrabactin and quinabactin, antagonists AA1 and RK460, and a group of analogues, PBIs (Ye et al. 2017; Ito et al. 2015; Benson et al. 2015). Quinabactin has ABA-like effects on e.g. biomass accumulation, and one of the analogues, PBI352, regulates stomatal aperture and drought tolerance, but does not affect germination or root growth (Okamoto et al. 2013; Benson et al. 2015).

1.1.2 Structural adaptations to stress

Plants have two central physical features that maintain internal water conditions and protect the plants from stress: leaf cuticle and stomata. The majority of transpiration is estimated to occur through stomata in water-saturated conditions and through cuticle under stress conditions (Suhita et al. 2004; Schuster et al. 2017).

Cuticle is a hydrophobic layer that covers the epidermal surfaces of plants, protecting the aboveground plant structures from drying, mechanical stress, UV radiation, and pathogens (Riederer 2006). It mainly consists of cutin (hydroxylated fatty acids) and wax (mostly very-long-chain fatty acids with various side groups), which are deposited on the surfaces of epidermal cells via ABC transporters, e.g. ABC11 (Fich et al. 2016; Bernard & Joubes 2013; Bird et al. 2007). Regulatory steps of cuticle formation are poorly understood, although some transcription factors that are involved in cuticle formation have been identified: MYBs 16, 30, 94, 96, and 106, HDG1, and WIN1/SHN1 are positive regulators, while MYB41 and DEWAX are negative regulators (Go et al. 2014; Seo et al. 2011; Cominelli et al. 2008; Lee & Suh 2015; Oshima et al. 2013; Wu et al. 2011). Cuticle formation is affected by environmental factors, such as water stress, which induces formation of a thicker cuticle in growing leaves (Suhita et al. 2004). How the environmental factors induce this change is largely unknown, except that ABA is crucial: ABA exposure increases cuticle wax content, while impaired ABA biosynthesis leads to cuticle deficiency

(L'Haridon et al. 2011). Additionally, MYB96, one of the positive regulators of cuticle formation, is induced by ABA (Seo et al. 2011).

The other major structures that regulate water transpiration and gas exchange in plants are the stomata, pores on the surfaces of plants surrounded by motile guard cells. The stomata open and close as the guard cells move depending on their turgor status. The movement occurs in response to changes in water status, and signalling-dependent changes in concentrations of several inorganic and organic ions (K^+, Cl^-, NO_3^-, $malate^{2-}$) and sugars.

Stomatal closure occurs through loss of turgor in guard cells caused by a massive efflux of K^+ and anions through specialized ion efflux channels on the plasma membrane. These channels are voltage-gated, meaning that they open when the membrane is depolarised (Joshi-Saha et al. 2011). The movement is induced by ABA, which activates the ABA signalling pathway that includes activation of the kinases SnRK2s, as detailed later in the thesis. SnRK2s phosphorylate and activate NADPH oxidase RBOHs F and D, triggering a ROS production event called respiratory burst. Respiratory burst stimulates the opening of Ca^{2+} channels, allowing influx of Ca^{2+} across plasma membrane (Kwak et al. 2003). This Ca^{2+} influx activates Ca^{2+}-dependent kinases (CDPKs and CIPKs) which, in turn, induce anion efflux channels (Tian et al. 2015; Zhang et al. 2014). Two types of anion efflux channels are involved in stomatal closure: slow (S-type, allows passage of NO_3^-, Cl^- and malate) and rapid (R-type). In Arabidopsis, the former is represented by SLOW ANION CHANNEL ASSOCIATED 1 (SLAC1) and SLAC1 HOMOLOG PROTEIN 3 (SLAH3), while the latter is represented by QUICK ANION CHANNEL ASSOCIATED 1 (QUAC1) (Schroeder & Keller 1992; Roelfsema et al. 2004; Geiger et al. 2011; Maierhofer et al. 2014). In addition to being activated through Ca^{2+} signalling, these anion channels can be activated Ca^{2+}-independently by multiple kinases, thus functioning as convergence points for several signalling pathways. Prolonged opening of the anion channels allows anions to escape from the cell along the electrochemical gradient, which depolarises the cell membrane and opens K^+ efflux channels, such as GATED OUTWARD-RECTIFYING K^+ CHANNEL (GORK) in Arabidopsis (Becker et al. 2003). This massive efflux of ions causes an increase in water potential in the guard cells, inducing water escape, loss of turgor, and closure of the stomata. In addition to inducing this Ca^{2+}-dependent movement of stomata, ABA also alkalises the cytosol (pH from 7.7 to 7.9), which stimulates the opening of K^+ efflux channels through a Ca^{2+}-independent pathway. This also causes further depolarisation of the plasma membrane by inducing inactivation of plasma membrane H^+-ATPase, represented by AHA1 in Arabidopsis (Virlouvet & Fromm 2015; Blatt 2000).

During stomatal opening, activation of efflux channels is inhibited and ions are transported back to the guard cells through uptake channels, such as K^+ CHANNEL IN ARABIDOPSIS THALIANAs (KATs) 1 and 2, as well as K^+ UPTAKE TRANSPORTERs (KUPs) 6, 8, and 2. This lowers the water potential within guard cells and induces water uptake, which in turn increases turgor and causes the stomata to open (Jezek & Blatt 2017). Other proteins that are involved in stomatal opening include e.g. nitrate transporter NRT1.1 (Guo et al. 2003).

Stomatal movements are regulated by multiple signalling pathways, where several phytohormones play important roles. In addition to its effects on kinases that regulate stomatal movement, ABA is also likely required for both methyl jasmonate -dependent and brassinosteroid-dependent stomatal closure (Yin et al. 2016; Kim et al. 2018). Salicylic acid triggers stomatal closure as well, even though in most cases it functions antagonistically to ABA (Zhao et al. 2017). Signalling gas NO, myrosinases TGG1/2, and kinases CPK3 and 6 have all been implicated in stomatal closure as well (Castillo et al. 2015; Islam et al. 2010; Mori et al. 2006). Emerging regulators of stomatal movement include strigolactones, which induce stomatal closure through SLAC1, independently of ABA; reactive carbonyl species, which are produced downstream of ROS production as the end products of lipid oxidisation; and CLE peptides (Lv et al. 2018; Islam et al. 2016; Zhang et al. 2018a). Stomatal movement can also be stimulated by other stress conditions, such as UV-B radiation, elevated CO_2, and ozone, all of which cause the stomata to close. Furthermore, blue light stimulates stomatal opening, and stomata close in response to pathogens (Suhita et al. 2004).

1.2 Protein kinases

Posttranslational modification of proteins is a convenient way of responding to environmental changes and regulating physiological activities, and thus common in signalling pathways, including stress signalling. Phosphorylation is among the most abundant modifications: protein kinase domains are common in genomes of eukaryotes, for example Arabidopsis has almost 1000 protein kinases, and by some estimations up to 50 % of cellular proteins are regulated through phosphorylation (Olsen & Mann 2013; Kaul et al. 2000; Kornev & Taylor 2010). Phosphorylation offers a rapid, inexpensive, and reversible way of controlling the activities of proteins.

Phosphorylation and its reverse reaction, dephosphorylation, are catalysed by kinases and phosphatases, respectively. Protein kinases catalyse the transfer of a γ-phosphate group (PO_4^-) from ATP to an amino acid. In prokaryotes, the phosphorylated amino acid is usually histidine, asparagine, cysteine, or glutamate,

or less commonly a serine, threonine or a tyrosine. Eukaryotic kinases transfer the phosphate group to a serine, threonine, or tyrosine.

Kinases contain multiple well-conserved domains that perform important functions (Hanks et al. 1988). One of these is a flexible structure called the activation loop or T-loop, which extends from subdomain VII to subdomain VIII of the catalytic domain. It is the most diverse segment in kinases, both in length and in sequence, but usually it contains a conserved His/Tyr-Arg-Asp (HRD) motif. Phosphorylation status of the activation loop frequently determines the phosphotransferase activity of the kinase. Based on structural comparisons of inactive and active protein kinases, phosphorylation within the activation loop induces conformational changes that stabilise it into a favourable orientation for substrate binding (Lai & Pelech 2016). Many eukaryotic protein kinases stimulate their activity by autophosphorylating residues within their activation loop. This autophosphorylation can occur intermolecularly between two of the same kinases ("*in trans*") or intramolecularly within one kinase ("*cis*") (Cabail et al. 2016). Activation loop can contain up to three phosphorylation sites, and some kinases do not have any.

Kinases have a well-organised interior, arranged around a single helix. The conserved core of a typical protein kinase consists of two lobes, a smaller, more flexible N-terminal lobe (N-lobe) and a larger C-terminal lobe (C-lobe) (Taylor & Kornev 2011). A conserved feature of the N-lobe is a catalytically important glycine-rich loop that covers the β- and γ-phosphates of ATP. The C-lobe contains binding sites for substrates and subunits, and both the activation loop and its neighbouring, cation-binding loop. A deep cleft between the two lobes forms the active site, where ATP localises. Prior to entrance to the active site, ATP binds to one or two divalent cations, either Mg^{2+} or Mn^{2+}, which compensate for the negatively charged phosphates of the ATP (Kornev & Taylor 2010).

1.2.1 SNF1/AMPK/SnRK1 kinase superfamily

Members of the SNF1/AMPK/SnRK kinase superfamily are known to participate in regulation of metabolism and stress responses (Hardie 2011). *Saccharomyces cerevisiae* SUCROSE NON-FERMENTING 1 (SNF1) is essential for release from glucose repression, a state where other metabolic pathways are repressed in the presence of glucose (Celenza & Carlson 1986). SNF1 is also required for carbohydrate accumulation, autophagy, nitrogen signalling, and various stress responses, including ER, Na^+, oxidative, alkaline pH, and antimycin A stress (Wang et al. 2001; Orlova et al. 2010; Mizuno et al. 2015; Hong & Carlson 2007). The

mammalian homologues of SNF1, AMP-ACTIVATED PROTEIN KINASEs (AMPKs), are activated in metabolic stress states where glucose is in high demand but of low supply. AMPKs inhibit energy-consuming processes such as protein and lipid biosynthesis, as well as cell growth and proliferation, and upregulate energy-producing processes such as glycolysis and fatty acid oxidation (Ghillebert et al. 2011; Hardie 2011). The Arabidopsis homologues of SNF1, SNF1-RELATED PROTEIN KINASEs (SnRKs), are involved in multiple pathways that govern energy balance and abiotic stress responses (Hrabak et al. 2003).

Both SNF1 and AMPKs consist of three subunits: a catalytic α-subunit (SNF1; or AMPKA), a non-catalytic scaffolding β-subunit (GAL38, SIP1, or SIP3; or AMPKB), and a regulatory/AMP-binding γ-subunit (SNF4; or AMPKG), which form a fully functional holoenzyme (Celenza & Carlson 1989; Crozet et al. 2014). SnRK1s also form heterotrimers, although their regulatory mechanisms are not similar to SNF1 or AMPKs, but whether SnRK2s or SnRK3s interact with functional β- or γ-subunits is not known (Emanuelle et al. 2015). Conservation of the superfamily is most evident in the catalytic α-subunit: SNF1, AMPK, and SnRK1 show approximately 62 % amino acid sequence identity in the catalytic subunit, and 48 % overall, while SnRK2s and SnRK3s have 42-45 % sequence identity to the α-subunit of SNF1, AMPK, and SnRK1 (Halford et al. 2004). Typical structures of the different members of the superfamily are shown in Figure 1.

Members of the SNF1/AMPK/SnRK superfamily are regulated in various ways, e.g. their target selectivity can be affected by the incorporated β-subunit. As is common for kinases, their activity is dependent on phosphorylation of the activation loop, which can occur through autoactivation and/or be catalysed by upstream kinases. SNF1 has three upstream kinases (SAK1/PAK1, TOS3 and ELM1) which activate it by phosphorylating it at residues Thr210 and Ser214, and several phosphatases, e.g. GLC7-REG1, that dephosphorylate and inactivate it (Estruch et al. 1992; Mccartney et al. 2016; Sutherland et al. 2003; Rubenstein et al. 2008). AMPK is phosphorylated by STK11/LKB1 and Ca^{2+}/CALMODULIN-DEPENDENT KINASE KINASE Beta (CaMKKBeta), which are homologous to the yeast upstream kinases, and by MAP3K7/TAK1 (Woods et al. 2003, 2005; Momcilovic et al. 2006).

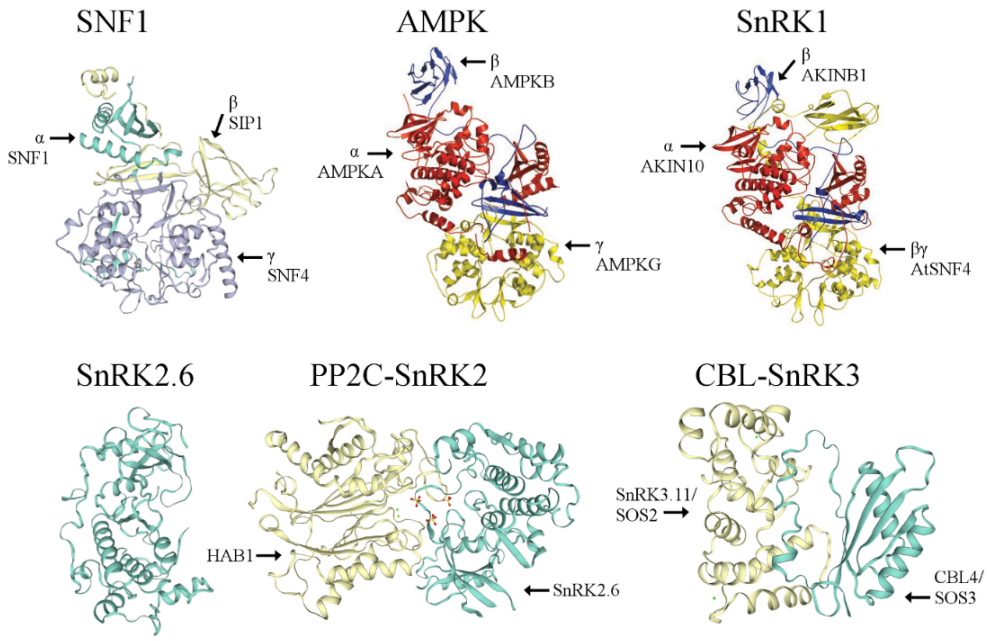

Figure 1. Structural comparison of members of the SNF1/AMPK/SnRK protein superfamily. SNF1, AMPK, and SnRK1 heterotrimers (upper row); SnRK2.6 in monomeric active form (lower left) and in inactive form in complex with a PP2C, HAB1 (lower middle); SOS2/SnRK3.11/CIPK24 in an active complex with a CBL, SOS3/CBL4 (lower right). Images were constructed according to crystallisations and modelling (Amodeo et al. 2007; Broeckx et al. 2016; Ng et al. 2011; Soon et al. 2012; Sánchez-Barrena et al. 2007).

1.3 SNF1-related protein kinase 1 (SnRK1) family

The SnRK1 protein family, which consists of three members in Arabidopsis (SnRK1.1/AKIN10, SnRK1.2/AKIN11, and SnRK1.3/AKIN12), are the closest plant homologues of yeast SNF1. Only the first two members of the family have been studied extensively; the third one is expressed predominantly in pollen and seeds and is considered to be less important for signalling than 1.1 and 1.2 (Schmid et al. 2005; Broeckx et al. 2016). SnRK1.1 and SnRK1.2 have distinct expression patterns, 1.1 being expressed broadly and 1.2 most abundantly in hydathodes, at the base of leaf primordia, and in vascular tissues (Williams et al. 2014).

Similarly to fully active SNF1 and AMPK, SnRK1s form heterotrimers that consist of α, β, and γ-subunits. They have three alternative β-subunits (AKINβ 1, 2, and 3) that are involved in selection of target specificity, and their functional γ-subunit AtSNF4 is unusual in that it contains a glycogen-binding domain typically found in β-subunits (Polge et al. 2008; Ramon et al. 2013). In addition to AtSNF4, there are several other candidate γ-subunits in Arabidopsis: KIN GAMMA 1

(KING1) is also capable of binding to the catalytic α-subunit, but it cannot complement the yeast γ-subunit mutant *snf4* (Bouly et al. 1999; Ramon et al. 2013). Additionally, KIN GAMMA 2 (KING2, At1g69800), At1g15330, and At1g80090 have 20-25 % homology to γ-subunits (Kushwaha et al. 2009; Robaglia et al. 2012). The latter two belong to the PV42 class of c-type subunits and are involved in reproductive development (Fang et al. 2011).

1.3.1 SnRK1s in signalling

Similarly to SNF1 and AMPK, SnRK1s play important roles in energy signalling (Baena-González et al. 2007). They function as central integrators of sugar, stress, metabolic, and developmental signals by controlling the functions of enzymes that are involved in various pathways. These enzymes include HMG-CoA REDUCTASE (HMGR), involved in sterol and isoprenoid production; SUCROSE PHOSPHATE SYNTHASE (SPS), regulator of sucrose synthesis; and NITRATE REDUCTASE (NR), involved in nitrogen assimilation (Robertlee et al. 2017; Sugden et al. 1999). SnRK1s are essential for repression of processes with high energy demand, such as protein synthesis (Nukarinen et al. 2016). In addition to regulating enzymes, SnRK1s induce massive transcriptomic reprogramming of a broad array of genes by regulating their transcription factors (Baena-González et al. 2007). These include WRINKLED1 (WRI1), a transcription factor that regulates glycolysis and lipid biosynthesis, and bZIP transcription factors (Zhai et al. 2017). Among bZIP transcription factors they regulate bZIP63, a key regulator of starvation responses, and bZIP11, an important regulator of carbohydrate metabolism and growth (Mair et al. 2015; Ma et al. 2011). bZIP11 regulates several genes related to signalling through Trehalose-6-phosphatase (T6P), a prominent growth regulatory molecule that accumulates in stress-adapted organisms and is also likely a direct negative regulator of SnRK1 activity (Ma et al. 2011; Zhang et al. 2009; Zhai et al. 2018).

SnRK1s have two upstream kinases, GEMINIVIRUS REP-INTERACTING KINASEs (GRIK) 1 and 2, also called SnRK1-INTERACTING KINASEs (SnAK) 2 and 1, that are capable of complementing a yeast mutant that lacks the SNF1 upstream kinases (Hey et al. 2007). The importance of GRIKs can be seen in how difficult it is to produce a double mutant plant line (*grik1-1grik2-1*), which is small in size, incapable of producing seeds, and requires sugar supplementation (Glab et al. 2017). GRIKs phosphorylate a threonine residue in the activation loop of SnRK1.1 and 1.2, and are in turn phosphorylated and inactivated by SnRK1s as part of negative feedback regulation (Shen et al. 2009; Crozet et al. 2010). Other forms of regulation that have been implicated in control of the activity of SnRK1s include

FCS-LIKE ZINC FINGER (FLZ) proteins, 5PTase13, N-myristoylation, and redox status (Carvalho et al. 2016; Jamsheer et al. 2018; Ananieva et al. 2008; Wurzinger et al. 2017; Pierre et al. 2007). Furthermore, degradation of SnRK1s is induced by ubiquitin E3 ligase PRL1 (Bhalerao et al. 1999) and by SUMOylation E3 ligase SIZ1. SUMOylation is possibly part of negative feedback regulation where SnRK1 activates its own degradation (Crozet et al. 2016).

SnRK1s regulate several stages of plant development, including seed maturation, vegetative growth, lateral organ development, and flowering (Tsai & Gazzarrini 2012; Chan et al. 2017; Jeong et al. 2015). They are important in dark adaptation, as well as responses to anoxia during submergence (Baena-González et al. 2007; Cho et al. 2016). SnRK1s are central regulators of the balance between growth and stress responses, since they interact with and probably regulate TARGET OF RAPAMYCIN (TOR) kinase, itself a central hub of metabolic and developmental processes. The two types of kinases are activated in stress or favourable conditions, with SnRK1s activating during a decrease in energy levels or sugar starvation, and TOR kinase activating under nutrient-rich conditions. The signalling pathways of SnRK1s and TOR are likely highly interregulated, which possibly includes direct interaction between the kinases, similarly to how AMPKs and TOR interact in mammals. It has been shown that SnRK1.1 can interact with (in vivo) and phosphorylate (in vitro) RAPTOR1B, a part of the TOR complex, suggesting that SnRK1s regulate the activity of the TOR kinase (Nukarinen et al. 2016).

1.4 SNF1-related protein kinase 3 (SnRK3) family

In addition to SnRK1s, which have close homologs in other organisms, there are two plant-specific SnRK families. SnRK3s, also known as CBL-INTERACTING PROTEIN KINASEs (CIPKs) or PROTEIN KINASE Ss (PKSs), are the largest SnRK family in Arabidopsis at 26 members. Similarly to other members of the superfamily, SnRK3s are activated by activation loop phosphorylation, but whereas other members of the superfamily have two phosphorylation sites in their activation loops, SnRK3s have three. In SOS2/SnRK3.11/CIPK24, phosphorylation mimicking mutation in any of the three sites (Ser156, Thr168 or Tyr175) increases the activity of the kinase *in vitro* (Gong et al. 2002). Uniquely among SnRKs, SnRK3s contain a C-terminal 21-amino-acid-long regulatory domain (FISL motif/NAF (Asp-Ala-Phe) domain) with a dual function. Removal of this domain increases the activity of SOS2, indicating that it is an autoinhibitory domain (Halfter et al. 2000), and on the other hand the domain is required for interaction with the Ca^{2+}-BINDING CALCINEURIN B-LIKE PROTEINs (CBLs), also known as SOS3-LIKE Ca^{2+}-

BINDING PROTEINs (SCaBPs) (Kim et al. 2000). Interaction with CBLs is required for release from the autoinhibitory effects of the NAF domain (Guo et al. 2002).

Arabidopsis has 10 CBLs with a shared 20-90 % sequence similarity (Luan 2009). They are monomeric when unbound (apo-form), but dimerise in response to Ca^{2+} binding (Sánchez-Barrena et al. 2005). Although Ca^{2+} binding is required for full activation of SnRK3s, at least some of them can be partially activated by CBLs in the absence of Ca^{2+} (Lin et al. 2014). CBLs can be divided into three groups based on the presence of Ca^{2+}-binding EF-hand sequences: group 1 (CBLs 1, 9) has two EF-hand sequences, group 2 (CBLs 6, 7, 10) has one, and group 3 (CBLs 2, 3, 4, 5, 8) has none (Batistič & Kudla 2004). The differences in Ca^{2+} binding are assumed to help in determining their specificities towards CIPKs, since CBLs function in complexes with different CIPKs in various pathways (Sánchez-Barrena et al. 2013). Subcellular localisation of each CIPK-CBL complex is determined by the bound CBL: CBLs that contain an N-terminal site Met-Gly-Cys-X-X-Ser-Lys/Thr that can be N-myristoylated at the Gly and S-acylated at the Cys are localised to the plasma membrane (CBLs 1, 4, 5, 9), while the others (CBLs 2, 3, 6, 7, 8, 10) are localised to the tonoplast (Cheong et al. 2007; Waadt et al. 2008; Batistič et al. 2010; D'Angelo et al. 2006; Sanyal et al. 2015; Batistič et al. 2012).

1.4.1 SnRK3s in signalling

In addition to requiring CBLs for activation, SnRK3s/CIPKs have putative upstream regulators that could affect their activity. Two MAP kinases have been identified as their potential upstream kinases, but the phosphorylation sites in CIPKs and the effects of this phosphorylation *in planta* are unknown (Popescu et al. 2009). Furthermore, clade-A PP2Cs can interact with CIPKs at either their phosphatase interacting motif (PPI) or at their N-terminal kinase domain, although also in this case the specifics of the interaction are not known (Lee et al. 2007; Ohta et al. 2003). What is known about regulation between PP2Cs and SnRK3s, however, is that CBLs (1, 2, 3, 5, and 7 but not 4, 6, or 9) can interact with and inactivate PP2Cs to recover CIPK-dependent AKT1 channel activity (Lan et al. 2011).

One of SnRK3s/CIPKs, SnRK3.11/SOS2/CIPK24, is central to the SOS (SALT OVERSENSITIVE) pathway, which is required for Na^+ stress tolerance. When vacuolar and apoplastic Ca^{2+} is released during Na^+ stress, it binds to and activates CBL4/SOS3 or CBL10/SCaBP8 (Lin et al. 2009; Zhu et al. 1998; Kim et al. 2007). The active CBLs bind to and activate SOS2, which in turn phosphorylates and inactivates the C-terminal autoinhibitory domain of plasma membrane Na^+/H^+ antiporter SOS1 at Ser237 (Quintero et al. 2011). This allows SOS1 to activate and pump Na^+ out of

the cell, maintaining low cytoplasmic Na^+ concentrations (Shi et al. 2000; Quan et al. 2007). The two CBLs, CBL4/SOS3 and CBL10, have different expression patterns and function mainly in roots and shoots, or leaves, respectively. It has been hypothesised that instead of helping to remove Na^+ from the cell, CBL10 is required for sequestration of Na^+ into vacuoles (Kim et al. 2007). Although SOS2 is its main regulator, SOS1 can also be targeted by MPK6, a kinase that is involved in the phospholipase D pathway (Yu et al. 2010). Activity of SOS2 is inhibited by 14-3-3 proteins λ and κ under non-saline conditions, likely through phosphorylation of Ser294 (Zhou et al. 2014).

SOS2 is also important for activation of other vacuolar transporters. These include tonoplast Na^+, K^+/H^+ exchangers NHXs, plasma membrane H^+/Ca^{2+} antiporter CAX1, and vacuolar H^+-ATPase. NHXs help in compartmentalising Na^+ into the vacuole and alleviating Na^+ cytotoxicity, CAX1 regulates Ca^{2+} fluxes and transport to vacuole, and vacuolar H^+-ATPase helps in building a transmembrane proton gradient that functions as fuel for transmembrane ion transport of e.g. Na^+ (Qiu et al. 2004; Cheng et al. 2004; Batelli et al. 2007). SOS2 is involved in ROS signalling by regulating the activities of RBOHF and H_2O_2 signalling molecules NDPK2, CAT2, and CAT3 (Verslues et al. 2007; Qu et al. 2017). Furthermore, SOS2 is a potential link to co-regulation of salt and ethylene signalling, since it phosphorylates and likely stabilises EIN3, an important transcription factor in ethylene signalling (Quan et al. 2017).

In addition to the known effects of SOS2/SnRK3.11/CIPK24, some functions of CBLs, CIPKs, and CBL-CIPK complexes have been characterised. Closely related CBLs 1 and 9 have been studied extensively: In complex with CIPK23/SnRK3.23, they regulate the activities of several ion channels, including K^+ TRANSPORTER 1 (AKT1), nitrate receptor/transporter CHL1/NRT1.1, and SLAC1, although in different phosphorylation site than its main regulator SnRK2.6 (Xu et al. 2006; Lee et al. 2007; Maierhofer et al. 2014; Held et al. 2011). They are also involved in ABA-dependent and independent osmotic stress responses in complex with CIPK1/SnRK3.16, and at least CBL1 is associated with cold responses in complex with CIPK7/SnRK3.10 (Pandey et al. 2004; Huang et al. 2011). Despite their high similarity, CBL1 and CBL9 have certain different functions, as CBL9, but not 1, is associated with regulation of ABA responses during seed germination in complex with CIPK3/SnRK3.17 (Pandey et al. 2004, 2008). Another pair of closely related CBLs, 2 and 3, affect plant growth, seed size, and embryonic development (Eckert et al. 2014). They are important in growth under high-Mg^{2+} conditions, possibly by aiding in vacuolar sequestration of Mg^{2+} in complex with CIPKs 3, 9, 23, and 26

(Tang et al. 2015). They are also involved in salt stress responses by localising CIPK21/SnRK3.4 to tonoplast (Pandey et al. 2015).

Of individual CIPK/SnRK3s, CIPK26/SnRK3.26 regulates the RING E3 ligase KEG, which is a negative regulator of ABA signalling that targets ABI5, ABF1, and ABF3 transcription factors for ubiquitination and degradation. CIPK26 can phosphorylate KEG, causing it to self-ubiquitinate and degrade, and reciprocally KEG targets CIPK26 for degradation (Lyzenga et al. 2017, 2013). CIPK6/SnRK3.14 is involved in salt tolerance, growth, and osmotic stress responses, and it targets K^+ transporter AKT2 to plasma membrane in complex with CBL4 (Tripathi et al. 2009; Chen et al. 2013; Held et al. 2011). CIPK8/SnRK3.13 is involved in early nitrate signalling, CIPK9/SnRK3.12 in potassium signalling, and CIPK11/SnRK3.22 in osmotic stress signalling (Hu et al. 2009).

1.5 SNF1-related protein kinase 2 (SnRK2) family

Members of the last SnRK family, SnRK2s, are known as central kinases in ABA-dependent and osmotic stress signalling, although they have also been implicated in other types of stress responses. There are a total of 10 SnRK2s, SnRK2.1-SnRK2.10, in Arabidopsis. They can be categorised to three groups based on their capacity to be activated by ABA: non-ABA-activated group I (SnRK2.1/2.4/2.5/2.9/2.10), weakly ABA-activated group II (SnRK2.7/2.8), and strongly ABA-activated group III (SnRK2.2/2.3/2.6). All SnRK2s, except 9, are additionally activated by osmotic stress through unknown mechanisms (Boudsocq et al. 2004). SnRK2s are not known to require subunits for activation, but they seem to be capable of forming heteromeres with each other (Waadt et al. 2015).

SnRK2s have two phosphorylation sites in their activation loops, only one of which is required for catalytic activity, but both of which are required for activation by osmotic stress or ABA. These are Ser175 and Ser171, respectively, in SnRK2.6/OST1 (Vlad et al. 2010). Other conserved features of SnRK2s include the SnRK2 box (Glu303-Pro318 in SnRK2.6) and ABA-box (Leu333-Met362 in SnRK2.6). ABA-box, and residues Ser7, Ser18, and Ser29, are critical for ABA signalling but not for function of the kinase (Belin et al. 2006). Activities of SnRK2s vary even among highly homologous members; for instance, SnRK2.6 has more efficient autophosphorylation, higher activity, and more stable structure than SnRK2.3 (Ng et al. 2011).

1.5.1 Components of the core ABA signalling pathway

SnRK2s are central kinases in the core ABA signalling pathway. In the absence of ABA, TYPE 2C PROTEIN PHOSPHATASEs (PP2Cs) lock SnRK2s into an inactive conformation where the activation loop of the kinase blocks its catalytic site, preventing it from activating via autophosphorylation (Ng et al. 2011; Yunta et al. 2011; Soon et al. 2012). This interaction occurs between strongly ABA-activated SnRK2s (2, 3, and 6) and PP2Cs, but also at least SnRK2.4 is dephosphorylated by PP2Cs (Krzywińska et al. 2016). When ABA is produced or released from internal storages, it binds to its receptors PYRABACTIN RESISTANCE 1 / PYR1-LIKE / REGULATORY COMPONENTS OF ABA RECEPTORs (PYR1/PYL/RCARs, hereafter PYR1/PYLs), activating them and enabling them to interact with PP2Cs (Ma et al. 2009; Park et al. 2009). This interaction detaches PP2Cs from SnRK2s, releasing them from repression. SnRK2s can subsequently phosphorylate their downstream targets, which include other enzymes and transcription factors (Umezawa et al. 2009; Vlad et al. 2009; Fujii et al. 2009).

The Arabidopsis genome contains 14 members of the PYR1/PYL ABA receptor family (Klingler et al. 2010). They belong to Bet v superfamily, structurally characterised by the START domain, which consists of a β-sheet flanked by two α-helixes (Radauer et al. 2008). PYR1/PYLs bind to PP2Cs through a so-called gate-latch-lock mechanism: ABA binds to a binding cavity in the receptor, inducing conformational changes that cause two β-loops to close over the molecule. The two loops consist of a proline "gate" Ser-Gly-Leu-Pro-Ala and a leucine "latch" Gly-Gly-(Glu/Asp)-His-Arg-Leu. Additionally, a "recoil" region (Met147-Phe159 in PYR1) aids in securing the closed gate. The closed loops provide a surface for the interaction between the receptor and PP2C, and a conserved tryptophan in PP2C inserts between the β-loops, locking the receptor in place. Contact between the receptor and phosphatase prevents access to the active site of the phosphatase (Melcher et al. 2009). Apart from activation by ABA, regulation of PYR1/PYLs is relatively unknown, except that at least some of them are phosphorylated by receptor-like kinase CARK1 and by TOR kinase (Zhang et al. 2018b; Wang et al. 2018). Additionally, multiple E3 ligases that target some PYR1/PYLs for degradation have been identified (RSL1, RIFP1, VPS23A, CRL4; Bueso et al. 2014; Li et al. 2016; Yu et al. 2016; Irigoyen et al. 2014). Certain PYR1/PYLs have been implicated in specific regulation: PYL8 and PYL9 play a role in ABA-mediated root growth, inhibiting at least ABI1/2, HAB1/2, and PP2CA/AHG3 and interacting with MYBs 44 and 77 (Antoni et al. 2013; Xing et al. 2016). Interestingly, there is evidence that PYR1/PYLs could repress function of non-ABA-activated SnRK2s, since a mutant of almost all

PYR1/PYLs is extremely insensitive to ABA, but its osmotic stress-induced activation of SnRK2s is enhanced (Zhao et al. 2018).

PP2Cs are monomeric Mg^{2+} and Mn^{2+}-dependent phosphatases that are primarily involved in stress signalling. There are 80 PP2Cs in Arabidopsis, arranged into clades A-K (Xue et al. 2008). The PP2Cs that have roles in ABA signalling belong to clade A, which contains 9 members: ABI1, ABI2, HAB1, HAB2, AHG1, PP2CA/AHG3, HAI1, HAI2, and HAI3. Of these all except HAI2 and 3 have been implicated in ABA signalling and/or as interactors of SnRK2s, although many of them also interact with other kinases, including SnRK1s and CIPKs/SnRK3s (Rodrigues et al. 2013; Fuchs et al. 2013; Antoni et al. 2012; Guo et al. 2002; Ohta et al. 2003; Lyzenga et al. 2013). Clade-A PP2Cs may also directly dephosphorylate some phosphorylation targets of SnRK2s (Lynch et al. 2012). In addition to inhibition by PYR1/PYL/RCARs, some upstream regulation mechanisms of PP2Cs have been identified: They participate in negative ABA feedback since the upstream promoter regions of PP2Cs contain elements for ABA-mediated induction (Bhalothia et al. 2016), and ABA-dependent transcription factors AtBH7 and 12, which also repress expression of PYR1/PYLs, induce expression of PP2Cs (Valdés et al. 2012). On the other hand, H_2O_2 inhibits function of HAB1, so they participate in positive ABA feedback as well (Sridharamurthy et al. 2014). Small GTPases, glutathione peroxidase, and FERONIA receptor kinase have also been implicated to affect activities of PP2Cs (Ludwikow 2015). Finally, at least some PP2Cs are targeted for degradation by PUB12/13 U-box E3 ligases and RGLG ligases 1 and 5 (Kong et al. 2015; Q. Wu et al. 2016).

1.5.2 SnRK2s in signalling

SnRK2s, mainly 2, 3, and 6, have several targets in the ABA signalling pathway. They control gene expression by phosphorylating transcription factors, such as ABA-RESPONSIVE ELEMENT-BINDINGs (AREB/ABFs; ABF1, ABF2/AREB1, ABF3, ABF4/AREB2, and ABI5), which bind to the ABA-responsive element (ABRE), a conserved *cis*-element present in promoters of many ABA-induced genes (Fujita et al. 2005; Furihata et al. 2006; Yoshida et al. 2015; Umezawa et al. 2013). Besides directly phosphorylating ABFs, SnRK2.2, 3, and 6 also phosphorylate and inactivate RAV1, a repressor of expression of ABFs 3-5 (Feng et al. 2014). Downregulation of transcription factor activity occurs through their ubiquitination and subsequent degradation. ABF2 is ubiquitinated by ARIA, ABI3 by AIP2, and ABI5 by KEG, DWA1, and DWA2 (Kim et al. 2004; Zhang et al. 2005; Liu & Stone 2010; Lee et al. 2010).

In addition to regulating transcription factors, SnRK2s control rapid responses to stress by regulating activities of several ion channels. SnRK2s, primarily SnRK2.6, facilitate stomatal closure by activating SLAC1 and QUAC1, and inactivating KAT1 (Imes et al. 2013; Nakashima & Yamaguchi-Shinozaki 2013; Geiger et al. 2009; Lee et al. 2009). Aquaporin PIP2;1 and vacuolar anion exchanger CLC, both of which contribute to stomatal movement, are also phosphorylated by SnRK2.6 (Grondin et al. 2015; Wege et al. 2014). Besides regulating channel activities, SnRK2.6 also regulates the expression of at least one channel: it phosphorylates bHLH transcription factor AKS1/FBH3, causing it to dissociate from DNA, which reduces expression of K^+ channel KAT1 (Takahashi et al. 2016). SnRK2s, mainly 2.6, also contribute to osmotic stress signalling by inducing ROS production through phosphorylation and activation of NADPH oxidases RBOHF and RBOHD (Kwak et al. 2003).

Among other ABA-related responses, strongly ABA-activated SnRK2s are crucial in maintaining seed dormancy. This can be seen in the triple mutant *snrk2.2/3/6*, which is insensitive to ABA during germination (Fujii & Zhu 2009). Although all three strongly ABA-activated SnRK2s, 2, 3, and 6, are crucial both for ABA responses during germination and stomatal movement, they have somewhat separate roles (Fujii & Zhu 2009; Fujita et al. 2009; Nakashima et al. 2009): SnRK2.6 is the major SnRK2 in control of stomatal movements, and it participates in cold responses by stabilising ICE1 and in primary metabolism by regulating oil synthesis, sucrose synthesis, and fatty acid desaturation (Ding et al. 2015; Zheng et al. 2010). SnRK2s 2 and 3, on the other hand, are important in maintaining seed dormancy, as well as for proline accumulation and root growth inhibition (Fujii et al. 2007). SnRK2.2 and 2.3 phosphorylate and inactivate SWI/SNF chromatin-remodelling ATPase BRAHMA (BRM), a protein that prevents activation of ABA signalling during non-stressed conditions by preventing expression of ABI5 (Peirats-llobet et al. 2016).

SnRK2s have been implicated in energy regulation through interaction with the TOR kinase pathway: TOR phosphorylates PYLs at a conserved serine residue in the ABA binding pocket (Ser119 in PYL1), which causes PYR1/PYLs to dissociate from the complex with ABA and PP2Cs. This inactivates ABA signalling to prevent activation of the stress response in unstressed plants. Under stress conditions SnRK2s phosphorylate RAPTOR, a part of the TOR complex, triggering dissociation and inhibition of the complex (Wang et al. 2018).

Even in the absence of ABA signalling, in a mutant *abi1-1* plant where interaction between PYR1/PYLs and PP2Cs is abolished by mutation at the PYR1/PYL binding site of the PP2C, all SnRK2s except 2.9 are activated by osmotic

stress through unknown mechanisms (Yoshida et al. 2006). This indicates that there are separate mechanisms for hyperosmolarity and ABA signalling.

In addition to PP2Cs, SnRK2s are inhibited by calcium sensor SCS and CK2, which negatively regulates at least SnRK2.6 by phosphorylating a cluster of conserved serines in the ABA box. This induces binding to PP2Cs and triggers protein degradation (Bucholc et al. 2011; Vilela et al. 2015). Ubiquitination and subsequent proteosomal degradation are important regulators of ABA signalling. Degradation of SnRK2s is generally poorly understood, but SnRK2.3 is degraded ABA-dependently through interaction with PP2-B11, part of an E3 ligase complex (Cheng et al. 2017).

SnRK2s are cross-regulated with other hormonal signalling pathways. This includes brassinosteroids, since BAK1 forms a complex with SnRK2.6 and BIN2 phosphorylates SnRK2.2 and 2.3, inducing ABA signalling (Cai et al. 2014). SnRK2.6 is also activated by brassinosteroid-regulated kinase CDG1-LIKE 1/PBS1-LIKE 7 (CDL1/PBL7), which it in turn activates through *in trans* phosphorylation (Kim et al. 2018). SnRK2s function antagonistically to gibberellins, since TYPE ONE PROTEIN PHOSPHATASE 1 (TOPP1) inhibits the activity of SnRK2.6 (Shang et al. 2016; Hou et al. 2016).

Among other subgroups of SnRK2s, SnRK2.8 is known to phosphorylate at least seven targets, including 14-3-3 proteins. Of the targeted proteins, glyoxalase I, detoxifies glycolysis byproducts and ribose-5-phosphate isomerase and catalyses a step in carbon fixation, linking SnRK2.8 to metabolic processes (Shin et al. 2007). SnRK2.8 also phosphorylates NTL6, a transcription factor whose target gene enhances dehydration resistance (Kim et al. 2012). SnRK2.4 and 2.10 bind to phosphatidic acid (PA), an important signalling lipid in stress responses (Julkowska et al. 2015), and they are important in maintenance of root architecture during salt stress (Mcloughlin et al. 2012). Additionally, SnRK2.10, at least, can phosphorylate dehydrins ERD10 and ERD14, and SnRK2.4 is involved in Cd stress responses (Maszkowska et al. 2019; Kulik et al. 2012). Multiple phosphoproteomic analyses have been completed for identification of targets of SnRK2s (Wang et al. 2013; Umezawa et al. 2013).

The involvement of SnRKs in stress signalling is summarised in Figure 2.

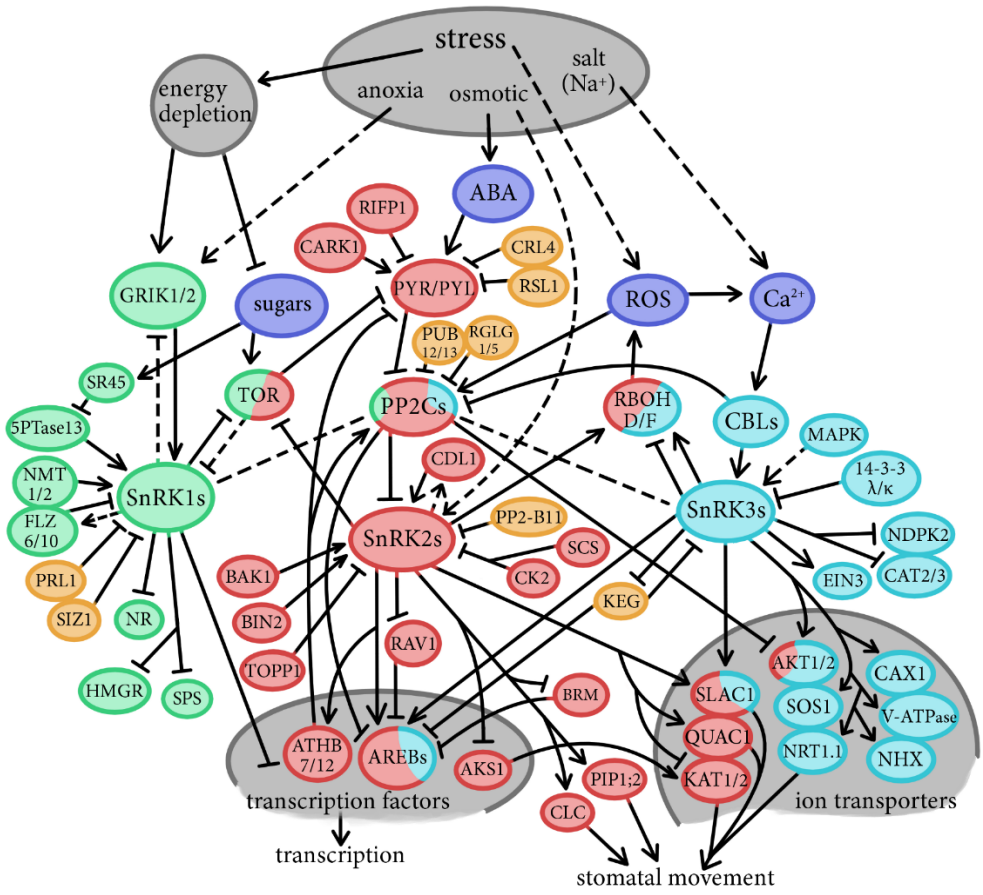

Figure 2. SnRKs in signalling. Pathways are marked for SnRK1 (green), SnRK2 (red), and SnRK3 (cyan). Multicoloured proteins indicate convergence points of several pathways. Non-protein signalling molecules are marked in purple, and E3 ligases of ubiquitination/SUMOylation - dependent degradation pathways are marked in orange.

1.6 Pladienolide B

One of the strategies of affecting plant behaviour is through chemical manipulation, which has been shown to be effective e.g. in manipulation of sugar signalling for improved grain yield and recovery from drought (Griffiths et al. 2016). Strong candidates for improving osmotic stress tolerance are ABA and its derivatives (Ito et al. 2015; Helander et al. 2016). However, ABA is an essential hormone in many developmental stages and functions of plants, meaning that applying the hormone would cause a wide range of effects in the plant. If precise regulation is desired, affecting targets downstream from signal initiation is necessary. A new candidate for behavioural control of plants, pladienolide B (Figure 3), is studied in this thesis. Pladienolide B is one of seven closely-related macrolides (pladienolides A-G)

produced by the bacterium *Streptomyces platensis* Mer-11107 (Mizui et al. 2003). Due to its strong tumour-inhibiting effects, it has been studied as a potential treatment agent for a variety of cancers, including chronic lymphocytic leukaemia and gastric cancer (Kashyap et al. 2015; Sato et al. 2014). Pladienolide B inhibits tumour-forming by arresting the cell cycle at the G1 and G2/M phases (Mizui et al. 2003). It binds to the SF3B1 factor of the splicing complex, disrupting splicing, which leads to production and nuclear export of intron-bearing precursors (Kotake et al. 2007). Pladienolide B functions as a splicing inhibitor also in plants, and it was found to activate the abiotic stress-associated ABA signalling pathway and disrupt the splicing of PP2Cs (Ling et al. 2017). The reduced amounts of functional PP2Cs might be the cause of activated ABA signalling, since PP2Cs inhibit SnRK2s. However, since drugs often have several targets (Hu et al. 2014), the possibility that pladienolide B might affect other components in the ABA pathway through a separate mechanism was investigated in this thesis.

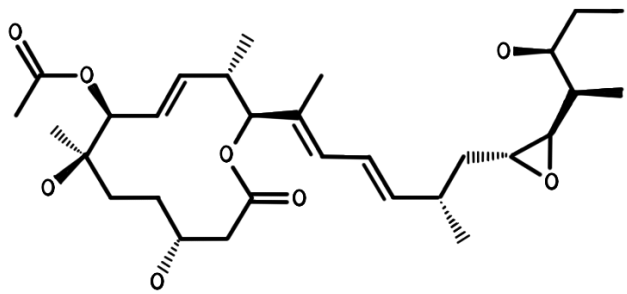

Figure 3. Molecular structure of pladienolide B. Structure retrieved from Protein Data Bank, ID: BGZ (http://www.rcsb.org/ligand/BGZ).

2 Aims of the Study

Plants adjust to their environments and the stress conditions that they face by utilising complicated regulatory signalling networks. If we want to improve productivity and tolerance of plants under stress conditions, it is important to characterise these networks. The SnRK protein kinase superfamily are central contributors to stress responses in plants, and due to their involvement in multiple signalling pathways, many of their functions probably remain to be discovered. The key objectives of this thesis were:

1. To find out whether and how potential regulatory proteins, KINGs, function in ABA signalling, and if they affect the functions of SnRK2s

2. To investigate if pladienolide B affects SnRK2s

3. To examine the effects of SnRK2s on cuticle formation

4. To analyse the behaviour of a newly constructed mutant of SnRK1 upstream kinases, GRIKs, that is suitable for analysing stress responses

3 Methodology

3.1 Biological materials

Arabidopsis thaliana accession Columbia-0 (Col-0; Rédei 1962) was used in all experiments. *A. thaliana* mutant *snrk2.6* has been described previously (Fujii et al. 2007). T-DNA insertion lines of the mutants for GRIK1, GRIK2, KING1 and KING2 (Salk_142938, Salk_015230, Sail_910_F09, and Sail_204_B03, respectively) were obtained from Arabidopsis Biological Resource Center (Sessions et al. 2002; Alonso et al. 2003). The homozygous insertion lines were identified by PCR screening.

3.1.1 Growth conditions

A. thaliana was grown in varying conditions depending on the target assay. For germination and root growth assays, seeds were sown on half-strength Murashige-Skoog medium plates (Murashige & Skoog 1962) supplemented with 1 % sucrose, stratified for 48 h at 4 °C in the dark, and grown at 23.5 °C, in 35 % relative humidity, with 16/8 h light/dark photoperiod and 75 μmol m^{-2} s^{-1} irradiance in either liquid or solid (1.2 % agar) medium. They were transferred to vertical plates after 4 days of normal growth for root assays, and grown for 4 additional days. For tests involving fully-grown plants, seeds were sown on 2:1 mix of peat and vermiculite, and grown at 23.5 °C, in 50 % relative humidity, with 16/8 h light/dark photoperiod and 100-150 μmol m^{-2} s^{-1} irradiance.

3.1.2 Plasmid constructs and mutagenesis

Several recombinant proteins were used in *in vitro* assays. Maltose-binding protein (MBP)-fused SnRK2.6 was described in Fujii et al. (2009). cDNAs for SnRK2.6, SnRK2.2, chimeric SnRK2.2-2.6, and KING1 were constructed by PCR, and cDNAs for mutated forms of SnRK2.6 were synthesized at Eurofins Scientific SE. Nucleotide exchange for mutated SnRK2.6s is given in the table below. Other constructs were cloned into BamHI-EcoRI of pGEX4T1 vector for production of glutathione-S-

transferase (GST)-fused proteins, while the chimeric SnRK2.2-2.6 was cloned into BamHI-SalI of pMALc2x vector for production of MBP-fused proteins.

Table 1. Nucleotide exchange for mutated forms of SnRK2.6

	amino acids		nucleotides	
residues	original	mutated	original	mutated
79-81	RFK	NLI	TAGATTCAA	AAATTTAAT
226-230	FRKTI	YKKIY	TCAGGAAAACTATA	ACAAGAAGATTTAC
61-65	NVKRE	VRREV	AATGTAAAAAGGGA	GTGCGCAGAGAAGT
44-46	NEL	GDN	AGTAATGAGCT	TCCGGAGACAA
46	L	N	CTT	AAC

3.1.3 Expression and purification of recombinant proteins

All recombinant proteins that were used in *in vitro* assays were produced in *Escherichia coli*. The plasmid constructs, described in the previous section, were transformed into *E. coli* Rosetta cells, and single colonies from the cells were grown overnight at 37 °C. The colonies were inoculated into and further grown in Luria-Bertani medium. Expression of the recombinant proteins was induced with 0.2 mM isopropyl beta-D-thiogalactopyranoside (IPTG), and after sufficient protein production, the cells were harvested by centrifugation at 4 °C. After resuspension into cold lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 100 µg/ml lysozyme) and 15 min incubation on ice, 50 mM dithiotheriol (DTT), 1 mM phenylmethanesulfonyl fluoride, and 1.5 % Triton X-100 were added, and solids were removed by centrifugation at 4 °C. Proteins were collected by incubating them with glutathione-agarose beads or amylose resin, depending on the fusion tag of the recombinant protein, for at least 1 h at 4 °C. Finally, the beads were washed with cold STE buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA).

The recombinant proteins were eluted from beads with 40 mM reduced glutathione (pH 8.0) for isothermal titration calorimetry and *in vitro* binding assays. After elution, the proteins were dialysed overnight at 4 °C in either STE solution for *in vitro* binding assays or phosphate buffer (pH 7.4, supplemented with 150 mM NaCl) for isothermal titration calorimetry assays.

3.2 *In vitro* kinase assays

Kinase activities were examined in *in vitro* kinase assays with recombinant proteins. Kinases were incubated with their substrates and other interacting proteins for 30-45 min at 30 °C in 20-40 µl of reaction mixture containing 25 mM Tris-HCl pH 7.4, 12 mM MgCl, 1 µM cold ATP, and 0.185 MBq [γ-^{32}P] ATP, with 1 mM DTT added for inhibition of proteases. In paper V, 20 mM Tris, 10 mM MgCl, 10 µM ATP, and 2 mM DTT were used instead. 1 mM Na_3VO_4 and 5 mM NaF were added for inhibition of phosphatases in papers I and III, 0.03 mg/ml bovine serum albumin was added for prevention of nonspecific reactions in paper I, and incubations were performed in the presence or absence of 2 µM pladienolide B in paper III. Reactions were stopped by adding Laemmli sample buffer (Laemmli 1970) and heat-treating the samples for 5 min at 70 °C, after which the proteins were separated by SDS-polyacrylamide gel electrophoresis. The radioactive protein bands were detected from dried gels by autoradiography.

3.3 Protein modelling

The protein complexes were modelled using structures retrieved from the Protein Data Base (PDB, http://www.rcsb.org). SnRK2.6-KING1 complex was modelled using the heterotrimeric AMPK complex (PDB id: 4CFE) as template, and SnRK2.6-pladienolide B complex was modelled with SnRK2.6 (PDB id: 3UC4) and pladienolide B (PDB ligand BGZ). The structure of pladienolide B was additionally retrieved from NCBI PubChem Database (PubChem id: 16202130).

Due to low sequence similarities between the catalytic AMPK subunit and SnRK2.6, and AMPK γ-1 subunit and KING1, both sequence and structural modelling were used to ensure optimal residue alignment of the SnRK2.6-KING1 complex. Sequence alignments were made with ClustalW and Multalin (Larkin et al. 2007; Corpet 1988).

3.4 Cuticle permeability assays

Cuticle permeability of *A. thaliana* plants was tested by examining the capacity of toluidine blue solution (TB, 0.05 %) for staining leaves (Tanaka et al. 2004). 5µl droplets of TB solution were placed onto leaves of 3-week-old plants, which were then covered for 2 h, or whole rosettes of 2-week-old plants were immersed in TB solution for 30 min. Plants were washed with water and photographed, and the sizes of the stains were measured from the photographs with ImageJ (https://imagej.nih.gov/ij/).

3.5 Gene expression assays

Gene expression in plants was evaluated with quantitative reverse transcriptase PCR (RT-qPCR). RNA was extracted from two-week-old *A. thaliana* with either GeneJET Kit (Thermo scientific) or TRIsure (Bioline) according to the manufacturer's instructions, and reverse transcribed from 1-5 µg of RNA. Diluted cDNA was then run in qPCR with HOT FIREPol EvaGreen (Solis Biodyne) or SYBR green (Bio-Rad) qPCR mix. YLS8, PP2AA3, TIP41, and Actin2 were used as reference genes in the various experiments. Relative amounts of RNA were calculated from the threshold cycles either directly with the ΔΔCt method (Livak & Schmittgen 2001), or by using them as input values in Qbase (Hellemans et al. 2007).

3.6 Western blotting

Relative protein amounts in plants were analysed with Western blot from two-week-old seedlings. In paper II, responses to ABA were examined after 3 h or 24 h treatment with 100 µM (±) ABA, and in paper III, responses to pladienolide B were examined after 30 min or 24 h treatment with 5 µM pladienolide B. Seedlings were collected and ground in liquid nitrogen, added to equal volume of Laemmli sample buffer (Laemmli 1970) and heat-treated for 5-10 min at 65-80 °C. Solids were removed by centrifugation, and the samples were run in SDS-polyacrylamide gel electrophoresis, followed by transfer to protein membrane (Immobilon-P, Millipore). Membranes were blocked with milk (Bio-Rad) and incubated with a primary antibody in TTBS buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 % Tween-20) over night at 4 °C with gentle agitation. After the primary antibody incubation, the membranes were incubated with HRP-conjugated anti-rabbit antibody in TTBS buffer for 1 h at room temperature in gentle agitation. The fluorescent signals were detected from the membranes with an ECL-based kit. For analysis of SnRK1 phosphorylation in paper V, anti-phospho-T172-AMPK-α antibody (Cell Signaling) and anti-SnRK1.1 (Agrisera) antibody were used for detecting phosphorylation of SNRK1s and amounts of SnRK1.1 protein, respectively. In paper II, KING protein amounts were analysed with anti-KING1 antibody (Agrisera). For analysis of FLAG-tagged protein amounts in paper III, 1 h incubation with HRP-tagged anti-FLAG antibody was sufficient for primary antibody incubation, and no secondary antibody was required.

3.7 Isothermal titration calorimetry

Isothermal titration calorimetry was used for further evaluation of interaction between SnRK2.6 and pladienolide B. The experiments were conducted with MicroCal 200 (Malvern) according to the manufacturer's instructions, at 20 injections/run, 30 °C cell temperature, 6 µCal/s reference power, 1000 RPM stirring speed, 0.4 µl injection volume 0.4 µl, and 120 s spacing. The protein and ligand were diluted in 4 % dimethylsulfoxide (DMSO), with respective concentrations of 0.04 µg/µl and 120 µM.

3.8 Statistics

All statistical analyses were carried out with either R (ver. 3.0.3) or SPSS data analysis program (ver. 24).

4 Overview of the Results

4.1 Interaction between recombinant KING1 and SnRK2s *in vitro*

SnRK2s are regulated by multiple proteins as part of different regulatory pathways, prominently in abiotic stress. Several other proteins in the SNF1/AMPK/SnRK superfamily form heterotrimers with β- and γ-subunits to activate, but it is not known whether SnRK2s can be regulated by subunits (Celenza & Carlson 1989). Canonical β- and γ-subunits have been identified for SnRK1s, but several other proteins with homology to the subunits have also been discovered (Ramon et al. 2013). It is possible that these homologs function as subunits for other SnRKs, including SnRK2s. One of the homologs is a potential γ-subunit KING1, which was identified as a putative regulatory component of ABA signalling during a screening study, where overexpression of KING1 lead to ABA insensitivity during germination. This suggested that KING1 is a negative regulator of ABA signalling (Papdi et al. 2008). In paper I, the roles of KING1 were studied further in regulation of ABA signalling as potential interaction partner of SnRK2s, mainly SnRK2.6/OST1. Direct physical interaction between recombinant KING1 and SnRK2.6 was detected in *in vitro* binding assay, as MBP-fused SnRK2.6 could be pulled down with GST-fused KING1 (paper I, Fig. 1A). Whether the interaction between the proteins was significant for the activity of SnRK2.6, and whether KING1 affected other SnRK2s, was examined by monitoring the kinase activities of SnRK2s in the presence of KING1 in *in vitro* kinase assay. KING1 was capable of suppressing the activity of SnRK2.6, reducing both its autophosphorylation and its capacity to phosphorylate a substrate, AREB1a (paper I, Fig. 1B). Intriguingly, the effects of KING1 on the activity of SnRK2.2 exhibited an opposite pattern: activity of SnRK2.2 was enhanced by KING1 *in vitro* (paper I, Fig. 1C).

4.1.1 Further insight into interaction between KING1 and SnRK2s

Interaction between KING1 and SnRK2s was investigated further by creating a model of the SnRK2.6-KING1 complex (Figure 4, paper I Fig. 2). The model was constructed on the basis of the crystal structure of human AMPK (PDB ID: 4CFE), which is a heterotrimeric complex of a catalytic α-2 subunit, a scaffolding β-1 subunit, and a regulatory γ-1 subunit. SnRK2.6 was aligned to the catalytic subunit, and KING1 to the γ-1 subunit. Only three amino acids in each protein had disallowed geometry, which was considered to have negligible influence on the final model. Interaction between the two proteins was predicted to occur between the C-terminal tails of KING1 and SnRK2.6, at amino acid residues 95-104 and 231-244, respectively. The C-terminus of SnRK2.6 contains two important segments that are conserved in SnRK2.2: the SnRK2 box (residues 303-317) and the ABA box (residues 333-362), which are crucial for activity of the kinase. The ABA box contains an α-helix that can function as a polar interaction surface for binding to other proteins (Yoshida et al. 2006; Ng et al. 2011).

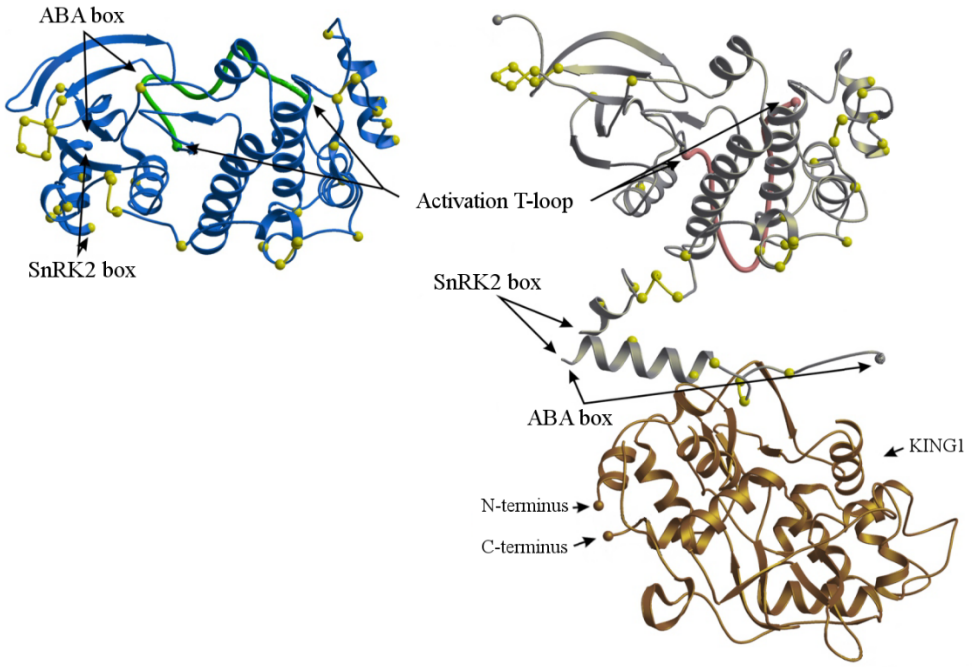

Figure 4. Modelling of interaction between SnRK2.6 and KING1. Inactive form of SnRK2.6 (left) and active form of SnRK2.6 in complex with KING1 (right).

The model for SnRK2.6, as described above, was constructed based on the active conformations of AMPK and SnRK2.6. However, inactive conformations of SnRK2.6 have also been reported at least twice (Ng et al. 2011; Soon et al. 2012). Both of these reports described two conformational changes in comparison to the active form: the C-terminal tail, which interacts with KING1, and the activation loop (residues 161-180) are turned towards the N-terminal activation site of the kinase, whereas in the active form they are oriented outwards. Considering these conformational changes in the C-terminal tail between the active and inactive form of the kinase, binding of KING1 to this region could cause conformational changes that resulted in changes of activity of SnRK2. This does not, however, explain how KING1 can influence the activities of SnRK2.2 and SnRK2.6 in different ways although the C-terminal binding site for KING1 is conserved between the two SnRK2s. How the different domains of the SnRK2s influence the ways in which KING1 regulates each SnRK2 were examined by observing the activity of a chimeric protein that consisted of the N-terminal part of SnRK2.6 and the C-terminal part of SnRK2.2. This chimeric protein could be purified although it was more degraded than either SnRK2.2 or SnRK2.6 (paper I, Fig. S4), but the corresponding chimeric protein consisting of C-terminal part of SnRK2.6 and N-terminal part of SnRK2.2 could not be produced in our conditions in *E. coli*. In *in vitro* kinase assay the chimeric protein behaved similarly to SnRK2.6, with reduced activity in the presence of KING1 (paper I, Fig. 1D). This indicates that the effects of KING1 on SnRK2s are influenced by the N-terminal part of the SnRK2.

4.1.2 Regulatory effects of KINGs *in vivo*

KING1 and SnRK2s were further examined *in vivo* in paper II. It is possible that multiple subunits are capable of regulating SnRK2s , as is the case with SNF1, AMPK, and SnRK1s, and thus other potential γ-subunit were considered as well. Among the other candidate γ-subunits, KING2 has the greatest homology to KING1 at 35.8 % identity, so it was examined alongside KING1 *in vivo* (Kushwaha et al. 2009; Robaglia et al. 2012). Mutant lines for the single *king* mutants and a double *king1king2* mutant were established for the analysis of the effects of endogenous KINGs *in vivo* (paper II, Fig. 1A). While none of the mutants exhibited unusual phenotypes under regular growth conditions (paper II, Fig. S1), and their transpiratorial water loss rates from leaves were comparable to wild type plants (paper II, Fig. 3), absence of either of the KINGs caused hypersensitivity to ABA during germination. The effect was similar in the single mutants and the double mutant, all of which germinated slower than wild type seeds in the presence of ABA (paper II, Fig. 2). The slow germination was

in agreement with the previous results by Papdi et al. (2008) which indicated KING1 as a negative regulator of ABA responses. Based on paper I, the influence of KINGs might be explained by their effect on the activities of SnRK2s. In addition to slower germination rates, the *king* mutants also exhibited slightly decreased post-germination root growth in the presence of ABA (paper II, Fig. S2). Although altered downstream effects on ABA signalling were not observed during ABA treatment of the mutants, in normal conditions the expression of one ABA-inducible gene (encodes xyloglucan endotransglucosylase/hydrolase protein 25, XTH25) was increased in the *king1king2* mutant in comparison to the wild type plants (paper II, Fig. 4). Interestingly, protein level expression of KINGs was also induced after 24 h treatment with exogenous ABA, further supporting their involvement in ABA signalling (paper II, Fig. 1C).

4.2 Pladienolide B can enhance the activity of SnRK2.6 *in vitro*

Knowledge of how SnRK2s could be manipulated chemically could be an advantage to helping plants survive under stress. The potential for chemical manipulation of SnRK2s was explored in paper III, where we investigated the capacity of the bacterial product pladienolide B to affect the activity of SnRK2s. Pladienolide is known as a splicing inhibitor in both mammals and plants, and in the latter it also activates core ABA signalling, possibly by preventing production of full-length PP2Cs (Ling et al. 2017). Further exploration of effects of pladienolide B on ABA signalling revealed that pladienolide B was capable of enhancing the activity of SnRK2.6, increasing both the autophosphoryation of recombinant SnRK2.6 and its capability to phosphorylate a substrate, AREB1a (paper III, Fig. 1), in *in vitro* kinase assay. Pladienolide B was incapable of inducing the same effect in SnRK2.2, indicating a selective activation of SnRK2.6 (paper III, Fig. S1).

The way in which pladienolide B affects SnRK2.6 was analysed by modelling their physical interaction. Two models were obtained for the interaction between pladienolide B and SnRK2.6. In model 1 pladienolide B binds to the cleft between the N- and C-terminal lobes, next to the activation loop, and in model 2, pladienolide B binds to the reverse side of the protein from the activation loop (Figure 5; paper III, Fig. 2). The veracity of the models and crucial residues for the binding were examined by modifying the amino acid residues of recombinant SnRK2.6 that should interact with pladienolide B in each model. The effect of pladienolide B on the kinase activities of these mutants was subsequently examined in *in vitro* kinase assay. Mutations at residues 61-65, 79-81 and 226-230 did not alter the effect that

pladienolide B had on the kinase, whereas mutation Asn-Glu-Leu → Gly-Asp-Asn at residues 44-46 abolished pladienolide B -induced activation of SnRK2.6. Upon further examination, mutation Leu46 → Asn was sufficient for this effect (paper III, Fig. 3). This mutation affects the binding site of pladienolide B according to the main docking of model 2, where Leu46 forms a hydrogen bond to one of the hydroxyl groups of pladienolide B (Figure 5, paper III Fig. 4). This indicates that model 2 shows the true binding between pladienolide B and SnRK2.6. The importance of the binding site could also be seen in isothermal titration calorimetry assay: binding-induced release of heat could be observed between pladienolide B and wild type SnRK2.6 or 226-230 mutant of SnRK2.6, but not the 44-46 mutant of SnRK2.6. (paper III, Fig. 4), although the K_d value for the binding could not be determined.

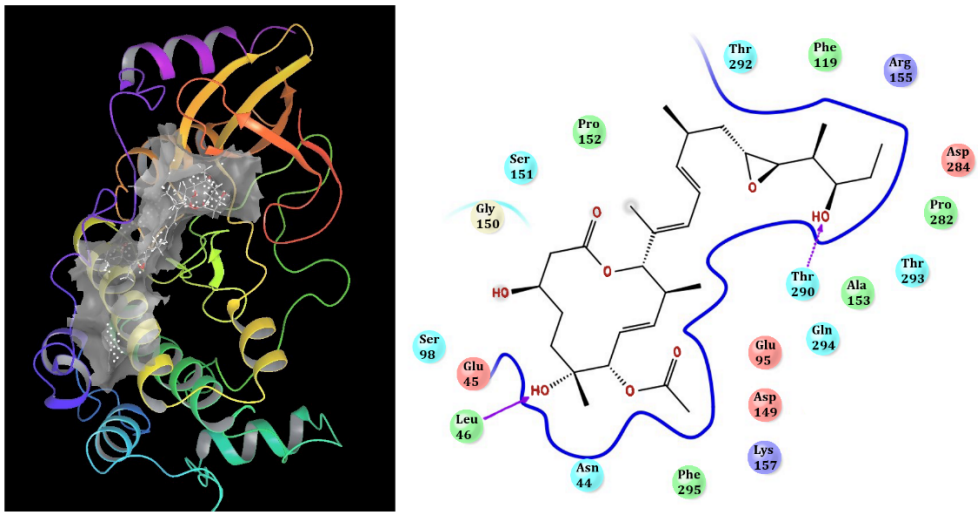

Figure 5. Modelling of interaction between pladienolide B and SnRK2.6. Model 2 of interaction between pladienolide B and SnRK2.6 (left) and interaction/docking map of the binding pocket of pladienolide B in SnRK2.6 (right).

In addition to enhancing the activity of SnRK2.6, pladienolide B can affect protein amounts of SnRK2.6. This was observed in mutant plants where SnRK2.6 was expressed under 35S promoter in an intron-free construct. Similarly expressed SnRK2.4 did not show changes in protein amounts, again indicating that pladienolide B shows a degree of specificity towards SnRK2.6 (paper III, Figure 5).

4.3 Core ABA signalling regulates cuticle formation

One of the known targets of ABA signalling is regulation of cuticle formation, which is crucial for prevention of water loss through surface transpiration. Cuticle also prevents the entry of pathogens, although some of them, such as *Botrytis cinerea*, can use it as a scaffold to infect the plant more easily. SnRK2s are central to ABA signalling, but whether they have an effect on cuticle formation was previously unknown. Moreover, the precise regulatory effects that ABA has on cuticle formation were uncharacterised. These were explored in paper IV, where cuticle integrities of various mutants that were deficient in ABA signalling were examined by testing whether the leaves could be stained in toluidine blue assay. Intact ABA signalling seems to be required for proper formation of the cuticle, since various ABA signalling deficient mutants had permeable cuticles, including both biosynthesis mutant *aba3* and mutants that are deficient in components of core ABA signalling, *pyr1pyl1pyl2pyl4pyl5pyl8*, *abi1-1*, and *snrk2.2/3/6*, but not other SnRK2s (paper IV, Fig. 2) (Gonzalez-Guzman et al. 2012; Fujii & Zhu 2009; Xiong et al. 2001). Curiously, the staining effect was strongest in the *snrk2.2/3/6* mutant. This suggests that cuticle formation is not wholly dependent on the upstream elements of ABA signalling, PYR1/PYLs and PP2Cs, but instead it has additional upstream regulatory elements of its own, and the signals are merged at SnRK2s. The mutant *abf2/3/4*, deficient in downstream elements of stress-related ABA signalling, on the other hand had intact cuticles. This suggests that the signalling pathway branches at SnRK2s, with stress-related ABA signalling and cuticle formation as separate downstream signalling pathways. The influence of the signalling pathway was also determined to be dependent on developmental stage, since young leaves were always free of stains. Moreover, formation of a thicker cuticle in the presence of low humidity was independent of both ABA signalling and SnRK2s (paper IV, Fig. 4). Together, these results indicate that the cuticle-ABA signalling network contains multiple branching pathways.

In addition to the cuticle permeabilities, the resistance of the various mutants to *Botrytis cinerea* infection was examined as well, and was determined to be increased in mutants with permeable cuticles and deficient ABA signalling. However, since *abf2/3/4*, a mutant with normal cuticle and impaired ABA signalling was not susceptible, the signalling pathways of both cuticle and *B. cinerea* resistance appear to branch away from ABA signalling pathway downstream of the core components, SnRK2s (paper IV, Fig. 6B and C).

4.4 GRIKs are important in glucose tolerance and activation of SOS2

The regulation of other members of the SnRK protein superfamily by upstream kinases of SnRK1s, GRIKs, was examined in paper V. Functions of GRIK1 and GRIK2 were studied *in vivo* in mutants *grik1-2*, *grik2-1*, and double mutant *grik1-2grik2-1*. Full-length mRNAs of the genes were not expressed in the mutants, but since a previous double knockout mutant of *grik1-1grik2-1* grew poorly (Glab et al. 2017) and the one used in this study grew and reproduced normally (paper V, Fig. 1C), it was hypothesised that the double mutant is a knock-down mutant with partially functional genes. Indeed, *grik1-2* mutant expressed an unusual mRNA that coded for an N-terminal truncated form of GRIK1 (paper V, Fig. 1E). The protein amounts and activation of GRIKs were analysed to evaluate the status of the SnRK1 signalling pathway in this new mutant. This examination was conducted with an anti-SnRK1 antibody, and an antibody that was raised against phosphorylated AMPK but recognizes phosphorylation in the activation loop of both SnRK1.1 and SnRK1.2 (Baena-González et al. 2007; Cho et al. 2016). Phosphorylation rates of both SnRK1s were reduced, but not eliminated, and total amount of SnRK1.1 was reduced in the GRIK double mutant *grik1-2grik2-1* (paper V, Fig. 1F). Lack of antibody against SnRK1.2 prevented analysis of its total amounts, but nevertheless the results show that GRIKs regulate both the phosphorylation rates and stability of SnRK1 proteins. The residual phosphorylation capability in the *grik1-2grik2-1* appeared to be sufficient to support its functionality, since the mutant grew both under normal conditions and in low-energy conditions, where SnRK1s are essential (Baena-González et al. 2007). This normal growth phenotype allows the use of the mutant for analysis of stress responses. In addition to the previously known glucose sensitivity, *grik1-2grik2-1* also showed increased sensitivity to NaCl (paper V, Fig. 2) which SnRK1s are not known to be involved in. This suggests that GRIKs could function in regulation of other pathways.

Certain members of the third SnRK family, SnRK3s/CIPKs, are involved in salt stress responses. The saline sensitivity of *grik1-2grik2-1* and the homology between SnRK1s and SnRK3s suggests that GRIKs could also be involved in regulation of SnRK3s. The connection between GRIKs and SnRK3s was examined in *in vitro* experiments with recombinant proteins. These experiments showed that SOS2 (SnRK3.11/CIPK24), a critical component of salt stress responses, could be phosphorylated and activated by GRIK1 (paper V, Fig. 3) at Thr168 of the activation loop. Moreover, GRIK1 was capable of activating SOS2 in a yeast reconstitution system (paper V, Fig. 7).

5 Discussion

Plants grow in constantly changing environments, rarely without being affected by stress in some way. Conditions with multiple simultaneous stressors present additional challenges, necessitating precise adjustment of responses for optimal survival and growth. Multiple studies have discovered common regulation mechanisms and points of convergence for different stress response pathways, but the signalling networks that links different stress signals, responses, and growth is complex and on the whole still poorly understood. Multiple proteins are known to have several functions in the network, possibly working as nodes for cross-regulation between multiple pathways.

SnRKs are an important kinase superfamily in abiotic stress regulation. They are central kinases in regulatory pathways of stress responses, such as ABA signalling, balance between energy preservation and growth, and salt stress responses. This thesis revealed several regulatory roles and functions of multiple members of the superfamily.

This work examined, firstly, KING1 and its homolog KING2, which are putative regulatory subunits of SnRKs. They were found to be capable of adjusting activities of the kinases *in vitro* (paper I), and affecting ABA-dependent germination, a pathway that SnRK2s are known to regulate (paper II). A chemical was found to be capable of regulating the activity of SnRK2.6 in paper III, and SnRK2s were examined in relation to cuticle formation in paper IV. GRIKs, upstream kinases of SnRK1s, were discovered to be important in NaCl tolerance, as well as being capable of targeting SOS2, a SnRK3, in paper V.

5.1 Effect of KING1 on activities of SnRK2s *in vitro* is influenced by the N-terminal region of the SnRK2

SnRK2s are regulators of multiple abiotic stress responses, and they have central roles in ABA-dependent stress responses. The strongly ABA-activated SnRK2s 2, 3, and 6, which are normally suppressed by clade-A PP2Cs, are released from inhibition when

ABA receptor PYR1/PYLs bind to and inhibit the phosphatases as part of the core signalling pathway (Umezawa et al. 2009; Vlad et al. 2009; Fujii et al. 2009). Homologs of SnRK2s, namely yeast SNF1, mammalian AMPK, and plant SnRK1s, are known to be fully active only as heterotrimers, which consist of a catalytic α-subunit and regulatory subunits β and γ (Celenza & Carlson 1989; Crozet et al. 2014). Whether SnRK2s require subunits to function, however, is not known. The role of a putative γ-subunit KING1 was investigated in paper I. Physical binding between SnRK2.6 and KING1 was observed *in vitro*, and kinase activity studies showed that KING1 could affect the activity of SnRK2s (paper I, Fig. 1). However, it had opposite effects on activity of two SnRK2s: it suppressed the activity of SnRK2.6, but enhanced the activity of SnRK2.2. The interaction between KING1 and SnRK2.6 was modelled based on the active trimeric form of the mammalian SnRK homologue AMPK, and the differences between active and inactive forms of SnRK2.6 were compared in order to hypothesise how KING1 affects the activities of SnRK2s. Two conformational changes were observed in SnRK2.6 based on our modelling and previously reported inactive forms of SnRK2.6 (Ng et al. 2011; Soon et al. 2012): firstly, in inactive form the C-terminal tail where the interaction with KING1 occurs is turned towards the N-terminal active site of SnRK2.6. Secondly, the activation loop of SnRK2.6 is orientated towards the SnRK2.6/KING1 interface in active form and towards the active site of SnRK2.6 in inactive form (Figure 4, paper I Fig. 2). Thus, binding of KING1 to the C-terminal region of SnRK2 could induce the conformational changes that cause the kinase to adopt the active conformation. This offers an explanation as to how KING1 induces the activity of SnRK2.2, similarly to the way that the γ-subunits regulate SNF1, AMPK, and SnRK1s (Ramon et al. 2013; Hardie 2011).

Although the model for binding between KING1 and SnRK2.6 clarifies the mechanism for activation of SnRK2.2, KING1 inhibits the activity of SnRK2.6 (paper I, Fig. 1). Since the causes for these disparate effects that KING1 has on SnRK2.2 and 2.6 are not readily apparent from the binding model, we analysed which regions of the SnRK2s determine how they are affected by KING1 from a chimeric mutant protein that consists of N-terminus of SnRK2.6 and C-terminus of SnRK2.2. The activity of the chimeric protein was suppressed by KING1, similarly to SnRK2.6, which indicated that the small differences in the N-terminal sequences of SnRK2.2 and SnRK2.6 apparently facilitate different interactions with KING1 (paper I, Fig. 1D). Previous research can support this conclusion, since the minor differences in the sequences of SnRK2s have been shown to influence the behaviour of the kinases. According to West et al. (2013), the N-terminal domains of SnRK2.6 and SnRK2.3 (which has 91 % identity to, and generally redundant functions with, SnRK2.2) have

different stabilities and protein conformations. SnRK2.6 is also a much more stable protein than SnRK2.2 or 2.3 in a thermoshift assay (Ng et al. 2011). It could be that KING1 binds to SnRK2.2 and 2.6 in a similar manner, but the greater stability of SnRK2.6 prevents it from inducing the conformational changes that would result in increased activity. A likely candidate for the differences in conformational changes is the activation loop, which adopts an open, flexible conformation in the active kinase and is locked into a more rigid conformation that obstructs the active site of the inactive kinase (Lai and Pelech 2016). Thus, when SnRK2.6 binds to KING1, the activation loop could get locked in its inactive state. Another unpredictable feature of the binding is the exact conformation of the N-terminus of KING1. In the binding model KING1 is aligned to the γ-subunit of AMPK, which is structurally unresolved in its N-terminus. The N-terminal part of KING1 is sufficiently large to potentially reach the interphase with SnRK2, which could influence the way in which KING1 affects SnRK2s.

5.2 KINGs are probable negative regulators of ABA signalling

The *in vitro* assays showed the potential for interaction and regulation between KING1 and SnRK2s. This is not, however, sufficient for confirming that this occurs *in vitro* in the same manner as *in planta*, where other interactors are also present. Additionally, KING1 could share redundant functions with its homologue, KING2, whose functions are unknown. Germination experiments in paper II showed that lack of either of the KINGs leads to slower germination in the presence of ABA. This agrees with the previously established result (Papdi et al. 2008) where overexpression of KING1 lead to ABA insensitivity during germination. On the other hand, post-germination roles of KINGs are difficult to discern, since no clear phenotypic differences could be seen between the *king* mutant and wild type plants (paper II, Fig. 3 and S1). The phenotypes are similar even under some stress conditions where SnRK2.6 plays an important role in stress signalling, although KINGs are expressed at post-germination developmental stages (Klepikova et al. 2015). On the other hand, ABA-treatment increases protein-level expression of KINGs in seedlings, indicating that the roles of KINGs during post-germination stages are likely connected to ABA-dependent signalling (paper II, Fig. 1). This accumulation of KINGs after exposure to ABA occurs relatively late, not being present after 3 h, which could indicate that KINGs function in late-stage adjustments to ABA-mediated responses. This could include e.g. control of leaf senescence. Furthermore, at least one ABA-dependent gene exhibits a different expression pattern in *king* double mutant than in wild type

lineage (paper II, Fig.4). The gene in question, *XTH25*, encodes a endotransglucosylase/hydrolase protein 25 that participates in cell wall construction of growing tissues (W. Xu et al. 1996). Since *XTH25* was more highly expressed in the absence of exogenous ABA in the *king* mutant, this indicates that KINGs could be negative regulators of ABA signalling.

Interpretation of the functions of KINGs, as well as the individual roles of KING1 and KING2, is difficult with the current data. In comparison to the *in vitro* experimental conditions in paper I, the situation *in planta* is more complex. Whereas only specific purified proteins are present *in vitro*, more interaction partners are available *in planta*, including inhibitors and cofactors or other binding proteins. Behaviour of the proteins can also be affected by fluctuating ion concentrations and pH. Moreover, suppressing or enhancing the activities of SnRK2s are not the only ways in which KINGs can affect ABA-dependent signalling; they could also affect e.g. ABA sensitivity or protein localization or concentration, possibly similarly to how CBLs aid in localisation of SnRK3s (Batistič et al. 2010). Considering the subtlety of the double KING mutant phenotype, it seems more likely that KINGs are not main effectors in ABA-dependent signalling pathway. The minor influence that KINGs have on the plant phenotypes could also be due to one of the other three putative γ-subunits having redundant functions with KINGs (Kushwaha et al. 2009; Robaglia et al. 2012), which could mask the effects on growth. It is also possible that KINGs have a role in specific conditions that were not studied during the experiments.

Rather than being major effectors, KINGs could function in cross-regulation between different pathways. They could also be involved in feedback regulation, considering their late accumulation after exposure to exogenous ABA (paper II, Fig. 1). Negative feedback regulation is a common mechanism in hormonal signalling, since strong reactions to adverse conditions can lead to excess suppression of growth and metabolism (Irigoyen et al. 2014; Zhang et al. 2017). This could indicate that KINGs have roles in long-term stress adjustment.

5.3 Activity of SnRK2.6 can be modulated with pladienolide B

Since functions of SnRK2s are central to multiple pathways in abiotic and biotic stresses, manipulating their functions in precise ways with e.g. chemicals could be a great asset in helping plants survive stress conditions. Unlike with regulation of SnRK2s by other proteins, not much is known about how they could be manipulated with the application of chemicals, other than ABA. Treatment with ABA derivatives

is one of the previously suggested methods of improving stress tolerance of plants, but ABA itself is not ideal for field conditions due to its instability and system-wide effects (Ito et al. 2015). If precise regulation is desired, affecting fewer targets is necessary; for example, affecting selected SnRK2s could allow control of specific downstream functions without compromising the functions of the whole plant.

Pladienolide B is a known splicing inhibitor in mammals, as well as in plants, where it also activates ABA signalling (Mizui et al. 2003; Ling et al. 2017). This activation occurs possibly through disruption of the splicing of PP2C mRNAs, which reduces activity in PP2Cs, negative regulators of ABA signalling (Ling et al. 2017). In paper III, we investigated whether pladienolide B had other effects on ABA signalling, and showed that it was capable of activating recombinant SnRK2.6. This could be done in an *in vitro* assay in the absence of PP2Cs, indicating that pladienolide B may affect SnRK2.6 directly instead of preventing its inhibition by PP2Cs (paper III, Fig. 1). The activation appeared to have high target specificity since it did not occur in the highly homologous SnRK2.2 (paper III, Fig. S1), which is promising when it comes to using pladienolide B to manipulate the activity of SnRK2.6, specifically. In comparison to ABA, which prevents inhibition of SnRK2.6 but does not enhance the activity of SnRK2.6 itself, pladienolide offers an intriguing research tool as an activator of SnRK2.6. In comparison to ABA, pladienolide B also seems to be more selective in targeting components of the ABA signalling pathway, potentially allowing for manipulation of a part of the signalling pathway, and bypassing the control by PP2Cs.

Modification of pladienolide B is necessary especially for in-field applications, since several of its properties make it unsuitable for immediate applications: it has poor solubility, and it is unstable and light-sensitive. Most of all, pladienolide B is harmful if applied to plants, since splicing inhibition affects many processes in plants, arresting plant development (Ling et al. 2017). The interaction between SnRK2.6 and pladienolide B was also elucidated in paper III through modelling of their binding. Experimental data suggested that a model where pladienolide B binds on the opposite side of the protein from the activation loop is likely to depict the true interaction (paper III, Fig.2 and 3). According to this model, pladienolide B forms hydrogen bonds with two of its hydroxyl groups to Thr290 and Leu46 of SnRK2.6. The interaction with the Leu46 residue appears to be significant to the binding, since a Leu46 → Asn mutation abolished the pladienolide B-induced activation of SnRK2.6 (paper III, Fig. 3). The precise map of interaction between pladienolide B and SnRK2.6 can provide possible sites where the chemical can be modified for future applications. This is especially important if pladienolide B should preferably interact with SnRK2.6 but not spliceosome, so as to prevent plant-wide adverse

effects of inhibited splicing. Comparing the interaction between pladienolide B and spliceosome, and pladienolide B and SnRK2.6, should be possible based on the results of this study and previous studies, which identified features that are important for the interaction and activity of pladienolide B, other small splicing inhibitor chemicals (FD-895, herboxidiene, spliceostatin A), and spliceosome (Effenberger et al. 2014; Kumar et al. 2016).

How pladienolide B would be applied on plants in the field should also be considered in possible modifications. The method of entry into the plant may be changed in soil compared to liquid-based cultivation systems, such as the one used in these studies, which commonly allow easier access of chemicals into plant root systems (Wu et al. 2015). Thus, access of the chemical to roots or leaves, as well as its capability to penetrate the tissues, should be evaluated further. Should the access of the chemical to the plant be difficult, and the chemical still highly unstable after potential modifications, a possible method of delivering the chemical to plants is production of the chemical at a root site. Production of ABA and its derivatives in modified bacteria in the rhizosphere has been previously suggested as a way to counteract their chemical instability and rapid catabolism, which might be possible also for pladienolide B or its derivatives (Ito et al. 2015; Xu et al. 2018).

In addition to its effects on activity of SnRK2.6, application of pladienolide B induced protein-level accumulation of FLAG-tagged SnRK2.6. This occurred in a plant line where the protein was expressed from a cDNA construct that lacked introns and was driven by a 35S promoter (Paper III, Fig. 5). The effects of pladienolide B on splicing of SnRK2.6 were presumably reduced, since there is no requirement for splicing in the intron-free construct. However, exactly how pladienolide B promotes expression of FLAG-SnRK2.6, but not SnRK2.4, is unclear. Pladienolide B could affect the protein in multiple ways: lack of splicing inhibition could contribute to protein accumulation, or pladienolide B could affect expression or stability of SnRK2.6 by binding to it. Binding of pladienolide B to SnRK2.6 is supported by our own *in vitro* data (paper III), and seems to be the most likely mechanism for avoidance of degradation. This could occur in several different ways: Binding of pladienolide B might directly stabilise SnRK2.6, but binding and subsequent increased activity could also lead to avoidance of degradation, since activity can affect degradation rates of proteins (Vilela et al. 2015; Castillo et al. 2015). The regulation of degradation by activity affects also the related SnRK1.1, although in its case inactivity of the protein leads to decreased degradation (Crozet et al. 2016).

5.4 Cuticle formation is guided by multiple distinct pathways

In addition to molecular-level changes, responses to stress also result in large-scale changes in plant morphology and growth. A critical aspect of responses to osmotic stress is the formation of cuticle in new tissues, which retains sufficiently well-watered conditions within the plants by limiting transpiration through the surfaces of leaves (Riederer 2006). ABA is known to be involved in cuticle formation, but previous studies indicated that only ABA biosynthesis mutants are defective in cuticle formation (Asselbergh et al. 2007; L'Haridon et al. 2011). The ways in which ABA signalling affects cuticle formation were investigated in paper IV. The morphologies of the sextuple ABA receptor mutant *pyrpyl112458* and mutants of other members of the major ABA signalling pathway, *abi1* and *snrk2.2/3/6*, indicated that also the major ABA signalling pathway is involved in cuticle formation (paper IV, Fig. 2). Examination of cuticle-associated transcription factors revealed several candidates for the endpoints of ABA-cuticle regulation pathway (paper IV, Fig. 3): *snrk2.2/3/6*, a mutant with defective ABA signalling, showed decreases expression of DEWAX, which is a negative regulator of cuticular wax synthesis, and increases expression of MYBs 16, 94, and 96, which are positive regulators of cuticle formation (Go et al. 2014; Lee & Suh 2015; Seo et al. 2011).

Although the main function of cuticle is thought to be protection from dehydration, it is also involved in other functions and signalling pathways, such as prevention of the entry of harmful agents and pathogens (Riederer 2006). Cellular mechanisms that are part of multiple functions commonly have several regulatory pathways that interact with each other and converge at some point, which appears to be the case also in cuticle formation. The regulatory pathways that affect cuticle formation apparently include distinct responses to abiotic stress, and on the other hand pathogens, cell death, and ROS, since expression of cuticle-promoting genes was increased during different abiotic stresses, but decreased in conditions that involved the other responses (paper IV, Fig. 6). Responses to the latter category were induced by *Botrytis cinerea*, which triggers immune responses and cell death; ozone, which induces apoplastic ROS formation and cell death; and ethylene, which can activate cell death signalling. Downregulation of cuticle-promoting genes could also be seen in the *acd11* mutant, which experiences a continuous ROS burst -dependent hypersensitive response (Govrin & Levine 2000; Overmyer et al. 2005; Brodersen et al. 2002; Cohn & Martin 2005). Furthermore, since cuticle genes are upregulated during drought in the ABA-dependent SnRK2 mutant *snrk2.2/3/6* (paper IV, Fig. 6),

there seem to be three branches of regulation of cuticle formation in response to stress: ROS/cell death, ABA/dehydration, and ABA-independent dehydration.

The high correlation between osmotic sensitivity and cuticle deficiency raises the question: how strongly is osmotic sensitivity an ABA-directed and stomata-mediated phenomenon, as it has traditionally been considered (Wang et al. 2011; Fujii, Verslues, and Zhu 2011)? The water loss phenotype of *snrk2.2/3/6* mutant, which has been assumed to be primarily due to transpiration through stomata, could be partially caused by increased water loss through the deficient cuticle (Fujii and Zhu 2009). This is supported by recent research in measurement of CO_2 concentrations inside the cells. Traditionally, the calculations for CO_2 concentrations are made with the assumption that water transpiration occurs mostly through the stomata, and gas exchange happens in parallel. However, according to Hanson et al. (2016) this overestimates the concentration of CO_2 inside the leaf by 15 % even under well-watered conditions. This indicates that a large portion of transpiration occurs through the cuticle, without simultaneous gas exchange. It is thus necessary to consider the effects of deficient ABA signalling by itself, as well as increased cuticle permeability, in mutants that could be relevant to both deficits. Caution should be exercised in regards to interpretation of ABA responses in mutants with changed ABA sensitivity, depending on how the change in sensitivity was identified.

One of the stresses where both ABA signalling and cuticle deficiency could play a role is *Botrytis cinerea* immunity, where ABA has been considered a negative regulator. Although cuticle generally prevents the entry of pathogens, the relationship between cuticle and fungal infections is complex: while a stiff surface can increase the required force for fungal penetration, soft surface abrasion, i.e. a defective cuticle, can also increase resistance to invasion (Benikhlef et al. 2013). Thus, the resistance to invasion was investigated in the various deficient mutants in paper IV. While the mutants in ABA signalling pathway (*aba3*, *pyrpyl112458*, *snrk2.2/3/6*) did resist invasion by Botrytis, the resistance correlates with cuticle permeability more closely than ABA insensitivity. This is most apparent if *snrk2.2/3/6* and *pyrpyl112458* mutants are compared: the former has a more permeable cuticle and the latter is more insensitive to ABA, but the former is more resistant to infection (paper IV, Fig. 6). The reduced correlation between resistance and ABA signalling can also be seen in lack of increased Botrytis susceptibility in ABA hypersensitive and hyperaccumulation mutants. All of this suggests that Botrytis resistance is primarily dependent on cuticle, rather than ABA signalling. Certain mutants are known to be involved in both Botrytis resistance and ABA responses. These include *botrytis susceptible 1*, *bos1*, and *zinc finger ankyrin repeat 1*, *zfar1*, both of which have increased susceptibility to Botrytis but as of yet unknown roles in ABA signalling,

although *bos1* mutation is known to enhance ABA-induced cell death (AbuQamar et al. 2006; Mengiste et al. 2003; Cui et al. 2013). This could provide a link between ABA regulation, cell death regulation and ROS signalling. All in all, dehydration-induced cuticle formation and ABA signalling in relation to humidity seem to form separate pathways. Although these results clarify the molecular mechanisms of cuticle formation, generalisations should be avoided since Arabidopsis has an atypical cuticle (Pollard et al. 2008).

5.5 GRIKs are essential for glucose tolerance

Activities of kinases are frequently regulated by upstream kinases, which have also been studied for SnRKs. Although inhibitors of SnRK2s are well-known, their upstream kinases (BIN2, CDL1) have been discovered only recently (Cai et al. 2014; Kim et al. 2018). SnRK1s, on the other hand, have been known for some time now to be regulated by upstream kinases, which are represented by GRIK1 and GRIK2 in Arabidopsis (Hey et al. 2007). In previous studies knocking out both GRIKs resulted in embryonic lethality, or in a sterile mutant that required sugar supplementation to grow beyond cotyledon-stage (Bolle et al. 2013; Glab et al. 2017). A new GRIK double mutant line was established and analysed in paper V. This mutant, *grik1-2grik2-1*, grew and produced seeds both in normal conditions and under dark and submersion (paper V, Fig. 1), conditions where activity of SnRK1s is required (Baena-González et al. 2007; Cho et al. 2016). The normal growth of the *grik1-2grik2-1* mutant is likely due to the GRIK1 allele, which produces a truncated protein that could retain some of its activity and provide sufficient support for seed viability. This hypothesis was supported by detection of residual phosphorylation at the activation loops of SnRK1s, which would usually be phosphorylated by upstream kinases (paper V, Fig. 1). The normal growth of the *grik1-2grik2-1* mutant under non-stressed growth conditions makes the newly established plant line a particularly suitable tool for analysing the effects of stress on plants.

Aside from residual activity of GRIKs in the *grik1-2grik2-1* mutant, it is also possible that SnRK1s are affected by other, unknown protein kinases. While there are no other close homologues of GRIKs in Arabidopsis, there are several other kinases that have a degree of homology to some of the upstream kinases of yeast SNF1 and mammalian AMPK. Yeast upstream kinases SAK1 and TOS3 are direct homologues of GRIKs, at approximately 35 % identity, but the third upstream kinase, ELM3, is more similar to CIPK22/SnRK3.19 and CIPK16/SnRK3.18, at 30 % identity (Estruch et al. 1992; Sutherland et al. 2003). Mammalian CaMKKs have up to 40 % identity to GRIKs, but the other upstream kinases are similar to other proteins in

Arabidopsis: STK11/LKB1 has 36 % identity to CIPK19/SnRK3.5, and MAP3K7/TAK1 has 38 % identity to protein kinase STY13 of unknown function and 37 % identity to uncharacterised putative kinase At4G1170 (Woods et al. 2003, 2005; Momcilovic et al. 2006). While interaction between SnRKs of the different families has not been reported, SnRK2s seem to be capable of forming heteromeres with each other, possibly to facilitate *in trans* autoactivation (Waadt et al. 2015). Thus, interregulation between the different SnRK families could be possible.

While the *grik1-2grik2-1* mutant exhibited a wild-type phenotype in most conditions, the double mutant was sensitive to high concentrations of glucose (paper V, Fig. 1), indicating that the potentially partially functional GRIK1 that is present in the mutant cannot fully replicate the functions of intact GRIKs. The absence of GRIKs in the mutant is likely to cause several effects on SnRK1-mediated pathways, including the observed decrease in the amount of SnRK1.1 (paper IV, Fig. 1).

5.6 GRIKs are capable of phosphorylating SOS2/SnRK3.11 and affecting salt stress responses

The GRIK double mutant *grik1-2grik2-1* showed increased sensitivity to NaCl, although SnRK1s are not known to be involved in NaCl tolerance (paper V, Fig. 2). This suggests that GRIKs have roles in regulation of multiple stress signalling pathways, and that they likely have more phosphorylation targets than just SnRK1s. Among SnRK1-related proteins, SnRK3s and in particular SOS2/SnRK3.11 are known to be involved in salt stress signalling (Shi et al. 2000; Quan et al. 2007). GRIK1 was found to be capable of phosphorylating SOS2, and of activating the reconstituted SOS pathway in yeast, but *grik1-2grik2-1* was less sensitive to salt stress than SOS2 mutant (paper V, Fig. 2-5). This indicates that other upstream regulation mechanisms, such as other upstream kinases, likely contribute to SOS2 activation.

6 Concluding Remarks

The research presented in this thesis contributes to our understanding of the regulation of plant stress responses. In particular, the roles and regulation of the SnRK superfamily of protein kinases were expanded in relation to ABA signalling, stress responses, energy signalling (Figure 6, left), and cuticle formation (Figure 6, right). The following conclusions were made:

- The activities of SnRK2s can be controlled by KINGs *in vitro*, and KINGs participate in ABA-related signalling *in vivo*
- Pladienolide B can activate SnRK2.6, creating the possibility for control of specific parts of SnRK-related signalling
- Cuticle formation is dependent on ABA-activated SnRK2s, but ABA and cuticle signalling pathways branch both up- and downstream from SnRK2s
- In addition to functioning as upstream kinases of SnRK1s, GRIKs participate in salt stress signalling, possibly by activating SnRK3s

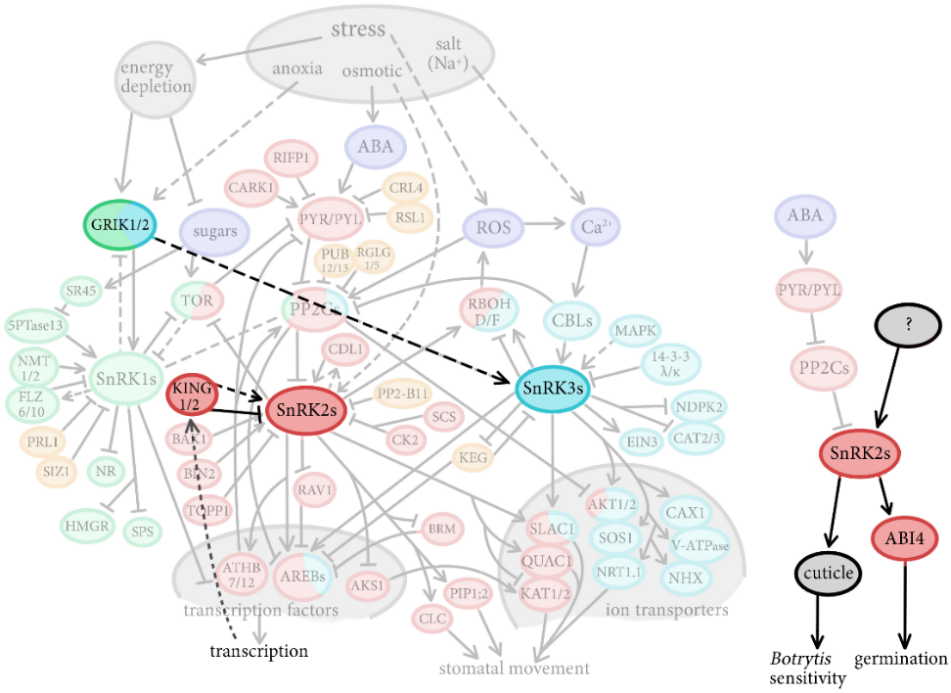

Figure 6. New regulatory roles of SnRKs. The newly discovered interactions between components of SnRK signalling pathways (left) and effect of SnRK2s on cuticle formation (right).

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