Regulation of plant energy signaling by components of the abscisic acid pathway

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“I speak to everyone in the same way, whether he is the garbage man or the president of the university”

Albert Einstein

Resumo

A capacidade de detetar e reagir a flutuações da disponibilidade de nutrientes é um aspeto crucial para a sobrevivência de todos os seres vivos. Em eucariotas, dois complexos proteicos altamente conservados evolutivamente, as proteinas cinases Snf1/AMPK/SnRK1 e TOR, desempenham um papel essencial no controlo homeostático energético. Condições de baixa energia activam o sistema Snf1/AMPK/SnRK1, que inicia assim uma reprogramação transcricional e metabólica que favorece os processos catabólicos (produtores de energia) sobre os processos anabólicos (consumidores de energia) com vista a restaurar a homeostase energética. Um dos alvos principais de Snf1/AMPK/SnRK1 é TOR, uma proteína cinase promotora de crescimento, que é inibida em condições de défice energético incapaz de sustentar o crescimento. Snf1/AMPK/SnRK1 e TOR constituem o eixo funcional Snf1/AMPK/SnRK1-TOR, que traduz o estado energético/nutricional celular em outputs de crescimento. A capacidade de detetar o estado energético permite ao eixo Snf1/AMPK/SnRK1-TOR reagir a diversas condições de stress que afetam o metabolismo energético primário.

Enquanto que em organismos unicelulares o eixo Snf1/AMPK/SnRK1-TOR responde principalmente à disponibilidade de energia e de nutrientes, com o aparecimento da multicelularidade este eixo evoluiu na capacidade de responder a sinais sistémicos, como hormonas e fatores de crescimento, coordenando respostas fisiológicas e crescimento de todo o organismo. Para além do seu papel nas respostas ao stress, o eixo Snf1/AMPK/SnRK1-TOR desempenha funções vitais na regulação do desenvolvimento.

Em plantas, o eixo SnRK1-TOR tem sido relacionado com respostas às principais fitohormonas, incluindo o ABA, mas de que forma essas vias estão interligadas a nível molecular é maioritariamente desconhecido. O trabalho apresentado nesta dissertação aborda a conexão entre SnRK1 e a sinalização por ABA, identificando dois componentes chave da sinalização por ABA como novos reguladores da via SnRK1.

Primeiro, as fosfatases PP2C clade A (reguladores negativos na via de sinalização por ABA) foram identificadas como necessárias para restabelecer a sinalização por SnRK1, com a falta destas fosfatases causando uma repressão defeituosa de SnRK1 quando os níveis energéticos são restaurado após stress. A repressão da activadade de cinase é exercida por ligação direta à subunidade catalítica α de SnRK1 através da desfosforilação e obstrução física. Como consequência desta regulação, a sinalização por SnRK1 é ativada por ABA numa forma dependente de PP2C.

Segundo, as cinases SnRK2 (reguladores poditivos na via de senalização por ABA) foram identificadas como repressoras da sinalização por snRK1 em condições normais de crescimento. As SnRK2 são necessárias para formar complexos SnRK1 repressores que respondem a ABA, mas não ao défice energético. Na presença de ABA, estes complexos contendo SnRK2 dissociam-se através da via de sinalização canónica por ABA, libertando SnRK1 e SnRK2 para desencadear respostas de stress e inibição de crescimento. Uma maior consequência desta libertação é a inibição de TOR e crescimento (crescimento pós-germinação e desenvolvimento de raízes laterais). Assim, SnRK2 desempenha um duplo papel na regulação de SnRK1: em condições controlo inibe a sinalização por SnRK1, contribuindo para um desenvolvimento e crescimento normais, enquanto em resposta a ABA atua concertadamente com SnRK1 para desencadear respostas ao stress e repressão de crscimento.

O trabalho apresentado nesta dissertação descreve novos mecanismos sobre a regulação do eixo SnRK1-TOR por ABA, adicionando conhecimento molecular sobre a modulação do ambiente no crescimento e desenvolvimento das plantas.

Abstract

The capacity to sense and react to fluctuations in nutrient availability is crucial for the survival of all living organisms. In eukaryotes, two highly evolutionarily conserved protein complexes, the Snf1/AMPK/SnRK1 and the TOR protein kinases, play an essential role in the control of energy homeostasis. Low-energy conditions activate the Snf1/AMPK/SnRK1 system, which thereby initiates a transcriptional and metabolic reprogramming to favor catabolic (energy producing) over anabolic (energy consuming) processes and ultimately restore energy homeostasis. One of the main targets of Snf1/AMPK/SnRK1 is the growth-promoting TOR kinase, which is inhibited in conditions of energy deficit that cannot sustain growth. Snf1/AMPK/SnRK1 and TOR constitute the Snf1/AMPK/SnRK1-TOR functional axis, which translates the cellular energy/nutritional status into growth outputs. The capacity to sense the energy status enables the Snf1/AMPK/SnRK1-TOR axis to react to a diversity of stress conditions that impinge on primary energy metabolism.

In unicellular organisms the Snf1/AMPK/SnRK1-TOR axis responds mainly to energy and nutrient availability while, with the onset of multicellularity, it evolved the ability to respond to systemic signals, like hormones and growth factors, to coordinate whole-organism physiology and growth. In addition to its role in stress responses, the Snf1/AMPK/SnRK1-TOR axis plays pivotal roles in developmental regulation.

In plants the SnRK1-TOR axis has been implicated in the response to the main phytohormones, including ABA, but how these pathways are interconnected at the molecular level is mostly unknown. The work presented in this thesis addressed the connection between SnRK1 and ABA signaling, identifying two core components of ABA signaling as novel regulators of the SnRK1 pathway.

First, clade A PP2C phosphatases (established negative regulators of ABA signaling) were found to be necessary to reset SnRK1 signaling, with lack of these phosphatases causing

defective SnRK1 repression when energy levels are restored after stress. Repression of kinase activity is exerted *via* direct binding to the SnRK1 α-catalytic subunit through dephosphorylation and physical obstruction. As a consequence of such regulation, SnRK1 signaling is activated by ABA in a PP2C-dependent manner.

Second, SnRK2 kinases (established positive regulators of ABA signaling) were found to act as repressors of SnRK1 signaling under normal growth conditions. SnRK2s are required to form SnRK1 repressor complexes that are responsive to ABA, but not to energy deficit. In the presence of ABA, on the other hand, these SnRK2-containing complexes dissociate through canonical ABA signaling, releasing SnRK1 and SnRK2 kinases to drive stress responses and growth inhibition. One major outcome of this release is the inhibition of TOR and growth (post-germination growth and lateral root development). Therefore SnRK2s play a dual role in SnRK1 regulation: in control conditions they inhibit SnRK1 signaling contributing to normal growth and development, while, in response to ABA, they act in concert with SnRK1 to drive stress responses and growth repression.

The work presented in this thesis describes novel mechanisms of how the SnRK1-TOR axis is regulated by ABA signals, contributing molecular knowledge into how plant growth and development are shaped by the environment.

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Chapter I – Introduction

Sessility and stress adaptation

All living organisms are constantly subjected to trophic and environmental pressures that determine the success or the failure of the individual and its species.

Motile organisms like metazoans can modify their behavior in relation to food availability or habitat favorability. In other words, animals can move seeking more, or better quality, nutriments or a more suitable environment for growing and, ultimately, for reproducing. On the contrary, plants are sessile and therefore they are constrained to live in the same *situ* where the seed germinates. This marked difference in the lifestyle between plants and animals reflects a profound difference in the evolutionary strategies between the two kingdoms which is also reflected in the generally higher degree of redundancy and size of plant genomes (Sterck et al. 2007, Panchy et al. 2016); in a simplistic view plants, more than animals, have to adapt to all the adverse conditions they encounter. Plants have evolved a myriad of pathways in order to perceive and respond to several environmental cues such as light, water availability, temperature, gravity, macro and micronutrient availability, but also pathogens, herbivores and chemical signals from other plants. All this information has to be finely integrated in order to achieve an appropriate physiological response. In the absence of a specialized apparatus like a central nervous system, the integration of multiple signals into a coordinated biological response represents one of the biggest challenges of plant life. This is true during “normal” development under favorable conditions, but becomes even more relevant when growth conditions are suboptimal.

Stress begins with a constraint (biotic or abiotic) or with highly unpredictable fluctuations that cause bodily injury, disease, or aberrant physiology (Gaspar et al. 2002). Hence, although the concept of stress is generally associated with the external factors that negatively affect growth or productivity, it primarily concerns the physiological alterations impairing the performance of a vital function (Gaspar et al. 2002).

The type of response to a stress situation is dependent on the nature and severity of the stressor, but also on the duration of the adverse condition. In the short term a stress condition elicits reversible plant responses in order to quickly adapt to the transient environmental challenge, while in the long term stress induces also developmental modifications or full growth arrest.

The ability of plants to modify their development in relation to environmental signals is defined as developmental plasticity and represents a pivotal feature of the plant's evolutionary success (Mizutani et al. 2018). Plasticity is defined as the ability of an organism to alter its physiology, morphology and development in response to environmental changes (Debat et al. 2001), and in plants is made possible by the modular and indeterminate mode of development, which occurs mostly post embryonically (Lachowiec et al. 2016, Mizutani et al. 2017). During embryogenesis only the basic body plan of the plant is established, including the main apical–basal axis that harbors the shoot and root apical meristems (Capron et al. 2009). These structures will give rise to all the above- and belowground organs, the identity, number, and characteristics of which will be largely determined by the environment that the plant encounters. Some examples of such responses include changes in root architecture to optimize water uptake, stress-induced flowering, changes in stomata density, but also plasticity at the molecular level, for example, transcriptional reprogramming (Lachowiec et al. 2016, Covarrubias et al. 2017, Mizutani et al. 2018).

In nature, plants are often exposed to several stress factors at the same time, and in some cases they trigger contrasting stress responses. For example heat stress is often linked to situations of drought. While heat induces stomata opening to decrease leaf temperature through transpiration, drought induces stomata closure to reduce water loss. Studies on *Arabidopsis thaliana* (Arabidopsis) and *Nicotiana tabacum* (tobacco) reveal that heat stress cannot trigger stomata opening when plants are simultaneously subjected to drought, resulting in a higher leaf temperature (Rizhsky et al. 2002, Rizhsky et al. 2004). Microarray analysis combining results from nine different abiotic stress

conditions (cold, osmotic, salt, drought, genotoxic stress, UV light, oxidative stress, wounding and heat) in Arabidopsis, let to the identification of 67 genes commonly responding to all these factors (Swindell 2006). Gene ontology analysis of these genes revealed that the functional categories overrepresented are related to cell rescue, defense, virulence and energy and metabolism (Swindell 2006). The latter, in particular, is in agreement with the idea that different stress factors converge on the impairment of energy metabolism and sugar signaling (Baena-Gonzalez et al. 2008).

Development is the result of coordinated cell growth and differentiation into specific tissues and organs. At the cellular level the decision to grow in response to an external stimulus needs to be balanced with the internally available energy resources to promote growth, or to restrain it. Two evolutionarily conserved protein kinase complexes are fundamental for plant energy management, the Sucrose non-fermenting-1 (SNF1)-related protein kinase 1 (SnRK1) and Target of Rapamycin (TOR) (Baena-Gonzalez et al. 2017). The functional relationship between these two kinase complexes serves as an important interface to integrate information on the nutrient and energy status with growth decisions at the cellular and organismal levels (Baena-Gonzalez et al. 2008, Hey et al. 2010).

1 Energy sensing by the Snf1/AMPK/SnRK1 complex

All organisms need to tightly adjust their physiology to the available energy resources. Evolution has shaped mechanisms to sense fluctuations around the optimal energy levels, converting them into signals able to trigger a whole set of molecular responses to restore energy homeostasis. A key player involved in both sensing and responding to conditions of energy deficit is the evolutionarily conserved protein kinase family that includes yeast SNF1, mammalian AMP-activated kinase (AMPK) and plant SnRK1 (Hardie 2007, Polge et al. 2007). These eukaryotic kinases act as heterotrimeric complexes composed of an α-catalytic subunit and two regulatory subunits, β and γ and they are all activated under conditions of low energy (Hardie 2007, Polge et al. 2007). Interestingly, the function of SNF1/AMPK/SnRK1 was proposed to be linked to the endosymbiotic acquisition of mitochondria, a fundamental step for the evolutionary affirmation of eukaryotes (Lane et al. 2010). It has been suggested that, as mitochondria became the main source of energy in aerobic metabolism, the SNF1/AMPK/SnRK1 kinase evolved as a system to sense the energy status of the cytoplasm and modulate mitochondrial metabolism (Hardie 2011). This hypothesis is corroborated by the genomic evidence that the fungus *Encephalitozoon cuniculi*, an obligate intracellular parasite, has lost both AMPK and mitochondria (Miranda-Saavedra et al. 2007).

In general, once activated by energy depletion, SNF1/AMPK/SnRK1 kinases induce an energy saving program by the coordinated upregulation of catabolic, energy-producing pathways and downregulation of anabolic, energy-consuming ones.

SNF1 exerts a fundamental role in the adaptation of yeast to use carbon sources alternative to glucose such as sucrose (from which derives the nomenclature Sucrose Non Fermenting 1), galactose and ethanol (Hedbacker et al. 2008). In glucose-rich conditions, yeast cells prefer a fermentative metabolism to grow, with the consequent release of ethanol to the growth medium. When glucose is not available, yeast cells undergo a drastic metabolic shift that allows the utilization of ethanol and other carbon sources that cannot be fermented. This involves the differential expression of more than 2000 genes,

out of which more than 400 respond specifically to SNF1 activation (Young et al. 2003). In addition to glucose limitation, SNF1 responds to factors such as alkaline pH, heat shock, toxic cations and antibiotics, among others (Hedbacker et al. 2008).

In mammals, aside to its role in cellular energy homeostasis, AMPK regulates energy metabolism at the whole body level. AMPK contributes to glucose homeostasis through the regulation of insulin production and glucose uptake by the skeletal muscle, but it also promotes lipid breakdown by modulating fatty acid oxidation in adipose tissue and it regulates appetite in the hypothalamus (Hardie et al. 2012). Systemic control of whole-body energy homeostasis by AMPK requires the action of hormones controlling glycemia, lipid breakdown, and body weight, but also other processes such as inflammation (Lim et al. 2010). AMPK activity in turn is regulated by several hormones in a tissue-specific manner; for example, leptin inhibits AMPK in the heart and the hypothalamus, whilst it activates it in the adipose tissue and the liver (Lim et al. 2010).

In plants, detrimental environmental conditions, such as drought, extreme temperatures, unexpected darkness, pollution and flooding, ultimately result in impaired energy production with consequent activation of SnRK1 signaling (Baena-Gonzalez et al. 2008, Tome et al. 2014, Mair et al. 2015, Nukarinen et al. 2016). Similarly to SNF1 and AMPK, SnRK1 activation triggers a vast transcriptional and metabolic reprogramming in order to balance energy homeostasis (Baena-Gonzalez et al. 2008, Tome et al. 2014).

1.1 Functions of SnRK1

The action of SnRK1 in energy metabolism is the result of the fine orchestration between the direct regulation of key metabolic enzymes with the transcriptional and translational initiation of an energy-saving program. Broadly, the onset of such energy-saving program is achieved by a coordinated downregulation of anabolism (energy-consuming processes) and upregulation of catabolism (energy-producing processes) (Baena-Gonzalez et al. 2007, Baena-Gonzalez et al. 2008, Tome et al. 2014).

Anabolic processes such as sucrose and isoprenoid biosynthesis, as well as nitrogen assimilation are repressed by phosphorylation of rate-limiting enzymes in spinach leaves (McMichael et al. 1995, Douglas et al. 1997), cauliflower (Ball et al. 1994) and Arabidopsis (Dale et al. 1995, Douglas et al. 1997). Early studies conducted *in vitro* reported SnRK1 is able to phosphorylate and inactivate 3-hydroxy-3-methylglutaryl CoA reductase (HMGR; isoprenoid biosynthesis), sucrose phosphate synthase (SPS; sucrose synthesis), and nitrate reductase (NR; nitrogen assimilation) (Sugden et al. 1999, Robertlee et al. 2017). The role of SnRK1 in the *in vivo* phosphorylation of such enzymes is supported by the observation that glucose-6-phosphate (G6P), known to repress SnRK1 (see section 1.3.3), represses also their phosphorylation (Toroser et al. 2000, Zhang et al. 2009, Nunes et al. 2013).

Other putative targets of SnRK1 are two glycolytic enzymes, pyruvate kinase (PK) (Beczner et al. 2010), the plant-specific non phosphorylated glyceraldehyde-3-phosphate dehydrogenase (NP-GAPDH) (Piattoni et al. 2011), and 6-phosphofructo-2-kinase/fructose-2-6 bisphosphatase (F2KP) (Kulma et al. 2004), which catalyzes the first irreversible step of sucrose production (Stitt 1987). In this way, SnRK1 contributes to the maintenance of a carbon/nitrogen balance through the coordinated regulation of carbon and nitrogen metabolism (Wang et al. 2012) . Finally, members of the TPS family belonging to the class II (like TPS5) are phosphorylated in a SnRK1-dependent manner (Glinski et al. 2005, Harthill et al. 2006). Although catalytically inactive, class II TPSs may potentially bind T6P or its sugar precursors and have been proposed to be involved in the regulation of carbon metabolism (Harthill et al. 2006, Ramon et al. 2009).

In some cases, phosphorylation by SnRK1 results in recruitment of 14-3-3 proteins, causing enzyme inactivation in the case of NR, F2KP and NP-GAPDH and, in the case of TPS5, potentially changing its signaling properties (Ikeda et al. 2000, Kulma et al. 2004, Harthill et al. 2006, Piattoni et al. 2011). 14-3-3 proteins are multi-functional proteins adaptors implicated in a growing number of cell biology metabolic processes and signaling, among which those dependent on TOR and AMPK (Kleppe et al. 2011).

In addition to direct enzyme phosphorylation, SnRK1 triggers a vast transcriptional reprogramming with more than 300 catabolism-related genes being upregulated and more than 300 anabolism-related genes being downregulated (Baena-Gonzalez et al. 2008). The action of SnRK1 on gene expression is partly achieved through direct phosphorylation of transcription factors such as bZIP63 (Mair et al. 2015, Droge-Laser et al. 2018). Phosphorylation of bZIP63 by SnRK1 changes its dimerization properties enabling differential gene expression (Mair et al. 2015). An over represented class of genes induced by the SnRK1-bZIP axis relates to proline and branched-chain amino acid metabolism (Mair et al. 2015) which can be used as alternative energy sources in the TCA cycle during carbon starvation (Szal et al. 2012). Interestingly, the expression of several class II *TPS* genes responds to SnRK1 activation and to sugar provision (which repress SnRK1 signaling). *TPS5* is induced by sugars and conversely repressed by SnRK1 activation whereas *TPS8-9-10* are repressed by sugars and induced by SnRK1 signaling (Price et al. 2004, Baena-Gonzalez et al. 2007, Osuna et al. 2007).
SnRK1 also represses gene expression in a miRNA-dependent manner (Confraria et al. 2013); however, the mechanisms underlying SnRK1 regulation of the miRNA pathway are thus far unknown.

A similar function to AMPK as whole-body energy regulator is also present in plants where SnRK1 is linked to regulation of carbon partitioning between source and sink tissues (Halford et al. 2003 Lin et al. 2014, Yu et al. 2015). In potato tubers SnRK1 regulates sugar/starch metabolism as well as tuber development (Lovas et al. 2003, McKibbin et al. 2006) whereas in pea seeds it affects reserve accumulation (Radchuk et al. 2006, Radchuk et al. 2010). In rice, the signaling of sugar demand from the germinating embryo (sink tissue) to the endosperm (source tissue) is mediated by SnRK1, which induces the expression of enzymes necessary for starch mobilization from the endosperm (Lu et al. 2007, Lin et al. 2014). In lower plants, such as bryophytes, SnRK1 is essential for mobilizing starch during the night. In the moss *Physcomitrella patens*, the double knockout of *SNF1a* and *SNF1b* (the genes encoding the SnRK1 catalytic subunit), is only viable if supplemented with exogenous glucose or if grown under a constant light regime

(Thelander et al. 2004). A similar impact on starch degradation was found in Arabidopsis in which virus-induced gene silencing of both SnRK1α codifying genes resulted in aberrantly high starch levels at the end of the night (Baena-Gonzalez et al. 2007). Similarly to AMPK, SnRK1 may also play tissue-specific functions and its effects may largely differ in source and sink organs. For example, overexpression of the catalytic subunit causes a decrease in glucose-induced starch accumulation in Arabidopsis seedlings (Jossier et al. 2009), whilst it results in increased starch accumulation in potato tubers (McKibbin et al. 2006).

Besides their role as an energy source, sugars act as universal signals regulating plant growth and development (Lastdrager et al. 2014, Li et al. 2016). Sugar signals interact with other environmental, hormonal and metabolic cues under normal conditions and during stress to shape a wide range of developmental processes from germination to flowering and senescence (Bolouri Moghaddam et al. 2013, Lastdrager et al. 2014, Cho et al. 2018, Martínez-Noël et al. 2018). As a central energy sensor (Baena-Gonzalez et al. 2008, Tome et al. 2014), SnRK1 is thought to be crucial for the integration of these signals into adequate growth and developmental decisions (Tsai et al. 2014, Baena-Gonzalez et al. 2017).

One particular pathway with which sugars have long been known to interact genetically is ABA signaling (reviewed in (Rolland et al. 2002). More recent work established links between ABA and SnRK1 signaling, in particular during seed development, germination and seedling establishment. Plants overexpressing SnRK1α1 show delayed germination and are hypersensitive to ABA during germination and early seedling development (Jossier et al. 2009, Tsai et al. 2012). In contrast, silencing of SnRK1 in pea seeds results in phenotypes reminiscent of ABA insensitivity, such as reduced accumulation of reserves and defective dormancy (Radchuk et al. 2006, Radchuk et al. 2010). These defects can be partly explained by reduced ABA accumulation and by decreased expression of *ABI3* and *FUSCA3*, encoding key transcription factors for embryogenesis, seed maturation, desiccation tolerance, dormancy, and germination (Devic and Roscoe 2016, Fatihi et al.

2016). In addition, proper regulation of seed maturation by ABI3 requires interaction with bZIP53 and bZIP10/bZIP25 (Alonso et al., 2009), dowsntream effectors of SnRK1 signaling (Droge-Laser et al. 2018). Besides transcriptional regulation, SnRK1 regulates FUSCA3 by direct phosphorylation, promoting its stability through the inhibition of proteasomal degradation (Tsai et al. 2012, Chan et al. 2017). FUSCA3 controls the embryonic-to-vegetative phase transition but has also an impact on the shift from vegetative to the reproductive state (Lumba et al. 2012). By controlling FUSCA3, SnRK1 was shown to act as a positive regulator of ABA signaling and as a general antagonist of developmental phase transitions (Tsai et al. 2012, Chan et al. 2017). For example, SnRK1 overexpression delays flowering under long days (Baena-Gonzalez et al. 2007, Tsai et al. 2012, Williams et al. 2014), and this phenotype can be rescued by the *fusca3-3* mutation (Tsai et al. 2012). Furthermore FUSCA3 phosphorylation by SnRK1 appears to be important for heat stress tolerance, impacting overall plant growth and fertility (Chan et al., 2017).
Additional connections between SnRK1 and ABA signaling include interactions of the downstream transcription factors during salt-induced metabolic reprogramming in roots (Hartmann et al., Plant Cell 2015) and *in vitro* phosphorylation of ABI5 and AREBP transcription factor peptides by SnRK1 (Zhang et al. 2008, Bitrian et al. 2011).

In this thesis, clade A PP2C phosphatases and SnRK2 kinases were identified as negative regulators of SnRK1 signaling, establishing two novel points of connection between these two pathways (Rodrigues et al. 2013)(Belda et al., - manuscript).

In addition to ABA, a crosstalk between SnRK1 signaling and phytohormones has been proposed for auxin, gibberellin, brassinosteroids, ethylene, jasmonate and cytokinin (Radchuk et al. 2010, Im et al. 2014, Nietzsche et al. 2016, Kim et al. 2017, Weiste et al. 2017, Simon et al. 2018).

The impact of SnRK1 on flowering is also dependent on the interaction with the circadian clock component cryptochrome 1 (CRY1) in response to nitrogen signals (Yuan et al. 2016). In mammals, AMPK regulates the circadian clock by phosphorylating CRY1 in the

nucleus, inducing its proteasomal degradation (Lamia et al. 2009). In plants nuclear SnRK1 activities appeared higher under high N conditions, and, as in mammals, an inverse correlation was observed between nuclear SnRK1 activity and nuclear CRY1 abundance , suggesting that in high N conditions SnRK1 delays flowering by inducing CRY1 destabilization (Yuan et al. 2016).

An additional factor reported to mediate the effect of SnRK1 on flowering is the Indeterminate Domain 8 transcription factor (IDD8), whose transcriptional activity is reduced by SnRK1 phosphorylation under sugar deprivation conditions (Jeong et al. 2015).

Mutations in the SnRK1 catalytic or regulatory subunits, affect also pollen development. Silencing of the SnRK1α subunit through antisense RNA results in morphological aberrations and male sterility in barley (Zhang et al. 2001), whereas βγ depletion precludes the rehydration of the pollen grain upon reaching the stigma (Gao et al. 2016). This defect was ascribed to a deficiency in mitochondrial and peroxisome biogenesis with a consequent impairment in ROS production and redox signaling (Gao et al. 2016) that is important for pollen-stigma communication (Traverso et al. 2013). RNAseq analyses of the *kinβγ* mutant pollen further showed reduced expression of an inward shaker K+ channel (SPIK) (Li et al. 2017). Mutants of this channel displayed defective pollen hydration on the stigma, suggesting that SnRK1 promotes pollen rehydration at least partly *via* SPIK (Li et al. 2017).

1.2 SNF1/AMPK/SnRK1 are heterotrimeric complexes

The high functional conservation of SNF1/AMPK/SnRK1 as energy sensors is reflected in structural similarities of the subunits and the αβγ heterotrimeric organization of the holoenzymes (Polge et al. 2007). The α-catalytic subunit is bound to β and γ regulatory subunits that modulate substrate recognition, intracellular localization and enzymatic activity in relation to cues on the cellular energy levels (Hedbacker et al. 2008, Hardie 2011, Crozet et al. 2014).

The broad level of functional conservation of SNF1/AMPK/SnRK1 is accompanied with plant specific differences for SnRK1 in terms of complex composition and regulation of kinase activity.

The gene families codifying for the various SnRK1 subunits have expanded in plants, diverging into plant-specific β and γ subunits that may form plant-specific SnRK1 complexes. Subunit isoforms appear to be expressed in distinct tissues, developmental stages, or in response to particular hormones or environmental conditions (Bradford et al. 2003, Buitink et al. 2004). In addition, processes like alternative splicing increase further the number of subunit variants and thereby the number of SnRK1 complexes that possibly exist (Gissot et al. 2006). The unique structure and regulation of SnRK1 may reflect the plant-specific lifestyle, particularly the more important role that sugars play in both signaling and metabolism in the carbon-fixing plants compared to the heterotrophic opisthokonts (Emanuelle et al. 2015).

1.2.1 α subunit

The α-catalytic subunit is codified by the Snf1 gene in yeast, by two genes in mammals (*PRKAA1/AMPKα1* and *PRKAA2*/*AMPKα2*) and by three genes in *Arabidopsis* (*SnRK1α1*, *SnRK1α2* and *SnRK1α3*). In plants, SnRK kinases expanded into a large family that comprises, in addition to SnRK1, the SnRK2 and SnRK3 kinases, with 10 and 25 members, respectively, in Arabidopsis (Hrabak et al. 2003, Halford et al. 2009). SnRK2s and SnRK3s are plant-specific, do not function as heterotrimeric complexes, and cannot complement *snf1* yeast mutant (Hrabak et al. 2003, Polge et al. 2007).

The α subunit is composed by two main domains: the catalytic or kinase domain (KD) at the N-terminus and the regulatory domain (RD) at the C-terminus. The catalytic subunit is the most conserved among species, reaching 62-64% amino acid identity when comparing the KDs of plant SnRK1α1 with yeast Snf1 and mammalian AMPKα (Halford et al. 2003). This domain folds in a canonical three dimensional structure (Hanks et al. 1995) that

contains the activation loop (T-loop) with a highly conserved Thr residue whose phosphorylation is essential for the enzymatic activity in all species (Hawley et al. 1996, McCartney et al. 2001, Baena-Gonzalez et al. 2007).

In yeast and mammals the reversible phosphorylation of the conserved residue in the T-loop represents one of the major, and best characterized, mechanisms of regulation of the kinase activity (see section 1.3). Both SNF1 and AMPK are phosphorylated by phylogenetically conserved upstream kinases, the yeast Sak1, Tos3, and Elm1 (Hong et al. 2003, Sutherland et al. 2003) and the mammalian LKB1 and CaMKKβ (Hurley et al. 2005, Woods et al. 2005). In Arabidopsis, two functionally redundant SnRK1 activating kinases have been identified: SnAK2 and SnAK1 (also named Geminivirus Rep protein-Interacting Kinases1 and 2, GRIK1 and GRIK2, respectively) (Shen et al. 2009, Crozet et al. 2010), which are also able to complement the yeast *sak1Δ tos3Δ elm1Δ* triple mutant (Hey et al. 2007). More details on the upstream kinases and their effect on SNF1/AMPK/SnRK1 are discussed in section 1.3.1.

The KD is followed by a three-helix domain referred as the autoinhibitory domain (AID) in AMPK and the ubiquitin-associated domain (UBA) in SnRK1. In opisthokonts the AID domain (residues 313-335 for AMPKα1) acts as an autoinhibitory sequence, as demonstrated by increased AMPK activity in deletion experiments conducted *in vitro* (Pang et al. 2007). In accordance with the poor conservation of this region (33-37%), the UBA domain plays different roles in SnRK1, promoting the phosphorylation by the upstream kinase SnAK1 and sustaining SnRK1 catalytic activity (Emanuelle et al. 2018).
The rest of the C-terminal region harbors the so-called kinase associated domain 1 (KA1) which is responsible for the interactions with the regulatory subunits (Kleinow et al., 2000; Crozet, Margalha et al., 2016). Moreover, as reported in Chapter II of this thesis, the KA1 might have additional regulatory functions since it seems to partly mediate the interaction with PP2C phosphatases (Rodrigues et al. 2013) and other regulators (Bhalerao et al., 1999; Farras et al., 2001). This phosphatase-interacting function for the plant KA1 domain is in accordance with previous findings from the structure of the

SnRK1-related SnRK3.11 (AtSOS2), containing a phosphatase interacting motif (PPI) in the KA1 domain that allows interaction with PP2C phosphatases (Ohta et al. 2003, Sanchez-Barrena et al. 2007).

1.2.2 β subunit

The β subunit plays an ancestral structural function, binding both α- and γ-subunits and thereby acting as a scaffold to keep the trimer integrity (Jiang et al. 1997, Iseli et al. 2005). Three main regions have been identified in all eukaryotes, the ASC (association with SNF1 complex), the KIS (kinase-interacting sequence), and the GBD (glycogen-binding domain) that, in AMPK, binds glycogen *in vitro* (Polekhina et al. 2005, Koay et al. 2010). The N-terminus contains a myristoylation site that regulates subcellular localization and kinase activity (Warden et al. 2001, Pierre et al. 2007, Oakhill et al. 2010). Myristoylation can affect kinase function by mediating the co-localization of SNF1/AMPK/SnRK1 with substrates or regulators (see section 1.3.2 on the protein phosphatases).

Yeast possesses three β subunits (Sip1/Sip2/Gal83), while mammals have two (β1 and β2). In higher plants, different numbers of β subunits have been reported: the Arabidopsis genome codifies for three (SnRK1β1, β2 and β3), while tomato and potato have four and two SnRK1β subunits, respectively. Arabidopsis SnRK1β1 and β2 present a typical domain organization, while β3 has an atypical domain architecture that appears to be restricted to the plant lineage (Gissot et al. 2004, Emanuelle et al. 2015). The β3 subunit contains a truncated KIS domain lacking GBDs and no N-terminal myristoylation site, but is able to complement yeast mutant lacking β function (Gissot et al. 2004). In analogy with AMPK, it has been proposed that β1 and β2, the plant β subunits harboring GBD domains, could potentially bind storage polysaccharides like starch which can be considered the plant analogue of glycogen (Hardie 2007). However, the results in this regard are highly controversial (see section 1.3.3 for details). Furthermore, the GDB domain of mammalian and plant β-subunits has several amino acid differences that could

eliminate important hydrogen bonds or hydrophobic contacts, or introduce a steric clash to prevent carbohydrate binding in the plant SnRK1β subunits (Emanuelle et al. 2015).

1.2.3 γ subunit and regulation by adenylates

It is well established that SNF1/AMPK/SnRK1 are activated in low energy conditions. The molecular proxy for energy availability in a cell is the energy charge of the adenylate system, namely the ratio between lowly charged adenylates, ADP and AMP, and the highly charged ATP (Atkinson et al. 1967). As the energy demand increases, or when energy production is impaired as a consequence of stress, the level of ATP declines whilst ADP and AMP rise, leading to the activation of SNF1/AMPK/SnRK1 complexes. The molecular mechanisms that allow the perception of energy imbalance and the subsequent activation of the kinase complex have been widely studied for mammalian AMPK, for which biochemical and physiological data are accompanied with structural evidence.

The adenylate charge is sensed by the γ subunit, which binds adenylates directly through the cystathione β-synthase (CBS) repeats (Cheung et al. 2000, Scott et al. 2004). The γ subunit of all eukaryotes contains at the C-terminal region four CBS repeats that, in pairs, fold in two structures called Bateman domains (Bateman 1997). The two Bateman domains assume a *head-to-head* conformation in which all the CBS sites form a ring structure (formed by CBS1/CBS2 and CBS3/CBS4) that functions as an energy sensing module (Scott et al. 2004). All CBS domains except CBS2 contain a conserved aspartate residue that binds the ribose ring of the adenosine, and therefore, only CBS1, 3 and 4 can be occupied by adenylates (Xiao et al. 2011, Chen et al. 2012). CBS1 and 3 are able to bind AMP, ADP and ATP in a dynamic and competitive fashion, and are considered the core of the adenylate sensing module. CBS4 was initially thought to bind exclusively AMP, but more recent reports indicate that, in some conditions it can also bind ATP, precluding the binding of AMP to CBS3 (Xiao et al. 2011, Chen et al. 2012). Crystal structures further show that the γ subunit binds the β subunit to keep the structural integrity of the αβγ

trimer, but that it also interacts with the linker peptide between AID and the C-terminal regulatory domains of the α subunit through the portion containing the CBS2 and CBS3 (Xiao et al. 2011, Xiao et al. 2013). Binding of AMP to the CBS3 site is proposed to induce AMPK activity by displacing the AID from the kinase domain and thereby by relieving its inhibitory effect (Xin et al. 2013). In general, binding of adenylates to the γ subunit is thought to affect the activation of AMPK by provoking structural rearrangements in the catalytic subunit with three different outcomes: i) stimulation of T-loop phosphorylation by upstream LKB1 kinases (Oakhill et al. 2011, Gowans et al. 2013); ii) inhibition of T-loop dephosphorylation by upstream phosphatases (Davies et al. 1995, Xiao et al. 2011); iii) allosteric activation (Gowans et al. 2013). Interestingly, i) and ii) can be triggered by both AMP and ADP (Oakhill et al. 2011, Xiao et al. 2011), although the ADP regulation of T-loop phosphorylation is controversial (Gowans et al., 2013), whereas iii) is only observed in response to AMP binding (Gowans et al., 2013).

Compared with mammalian AMPKγ, the yeast γ-subunit SNF4 presents fundamental structural differences, which reflect divergence in its mode of regulation. The yeast γ-subunit is able to bind adenylates, but the crystal structure reveals that the two Bateman domains form only one functional adenylate binding site in which AMP or ATP can be exchanged (Rudolph et al. 2005, Townley et al. 2007). Furthermore, SNF1 is not allosterically regulated by AMP (Wilson et al. 1996, Adams et al. 2004), and the reported effect of ADP in protecting the T-loop from dephosphorylation seems to be largely independent from the γ-subunit, and to rely instead on direct ADP binding to the Snf1 active site (Chandrashekarappa et al. 2011, Chandrashekarappa et al. 2013).

The γ subunit represents one of the most remarkable differences between plants and opisthokont AMPK-like complexes. In plants, three types of γ-subunit have been identified: canonical γ (Bouly et al. 1999), PV42/BsnIP1-type proteins (Slocombe et al. 2002, Fang et al. 2011) and βγ subunit (Kleinow et al. 2000, Lumbreras et al. 2001). The level of conservation among AMPKγ, SNF4, and plant γ-subunits varies between 20% and 35% and is largely due to the two Bateman domains, that are conserved in all species

(Polge et al. 2007). While the canonical γ subunits from tomato (*Lycopersicon esculentum*) and Medicago (*Medicago truncatula*) are able to complement the yeast *snf4* mutant (Bradford et al. 2003, Bolingue et al. 2010), the same is not true for the Arabidopsis BsnIP1 or SnRK1γ (Bouly et al. 1999, Slocombe et al. 2002), which is unable to interact with the β subunits in mature leaf cells (Ramon et al. 2013). By contrast, the βγ subunit of Arabidopsis functionally complements the yeast *snf4* mutant, interacts with the Arabidopsis SnRK1α and β subunits in yeast two-hybrid assays and assembles into plant-specific SnRK1 complexes *in planta* (Kleinow et al. 2000, Gissot et al. 2006, Lopez-Paz et al. 2009, Ramon et al. 2013). It has been reported that βγ could interact with SnRK1α without any β subunit, hypothesizing a unique dimeric constitution of certain SnRK1 complexes (Lopez-Paz et al. 2009). The nomenclature "βγ" derives from the domain organization of this subunit: in addition to the two Bateman domains at the C-terminus (with only 7% sequence identity compared with SnRK1γ), it harbors a GBD domain typical of β-subunits at the N-terminus (Lumbreras et al. 2001, Emanuelle et al. 2015). The conclusion that βγ is the *bona fide* γ subunit of Arabidopsis is further supported by high-resolution phylogenetic analyses, in which βγ and not γ appears to be monophyletic to the yeast and mammalian counterparts (Ramon et al. 2013). As further confirmation, Arabidopsis *snrk1γ* mutants do not show any obvious phenotypes, while, similarly to the lethality of the *snrk1α1 snrk1α2* mutant (Baena-Gonzalez et al. 2007, Ramon et al. 2019), *snrk1βγ* mutants are not viable, demonstrating that the βγ and not the γ subunit is indispensable during plant development (Ramon et al. 2013).

There is no structural information on the βγ subunit, but modeling the CBS of SnRK1βγ using the AMPKγ structure identified substitutions at two key aminoacids (Arg70 and His 151 in AMPKγ) involved in adenylate binding (Emanuelle et al. 2015). This may explain why SnRK1 complexes are not allosterically regulated by AMP (Sugden et al. 1999, Emanuelle et al. 2015). Furthermore, when introduced in AMPKγ, these mutations abrogate the AMP allosteric effect (Adams et al. 2004, Sanders et al. 2007). While AMP does not allosterically activate SnRK1, it could still positively impinge on the kinase activity. However, although earlier *in vitro* work showed that AMP protects the SnRK1α

subunit from dephosphorylation by PP2C phosphatases (Sugden et al. 1999), this effect could not be confirmed in subsequent studies (Emanuelle et al., 2015).

1.3 SnRK1 regulation

Reversible phosphorylation of the T-loop of the α subunit represents a major mode of regulation of SNF1 and AMPK (Stein et al. 2000). Several upstream kinases and phosphatases acting on the conserved T-loop threonine have been identified as positive and negative regulators, respectively (Hey et al. 2007, Mayer et al. 2011, Xiao et al. 2011, Crozet et al. 2014). In plants, the phosphorylation of the T-loop of SnRK1α is required for its activity (Baena-Gonzalez et al. 2007, Shen et al. 2009) but, contrary to yeast and mammals, the evidence correlating T-loop phosphorylation with SnRK1 activation is controversial. Although T-loop phosphorylation was shown to increase in response to hypoxia during submergence (Cho et al. 2016), no changes in T-loop phosphorylation were detected in other SnRK1 activating and deactivating conditions, raising the question of how SnRK1 activation occurs (Baena-Gonzalez et al. 2007, Fragoso et al. 2009, Coello et al. 2012, Rodrigues et al. 2013). Differential T-loop phosphorylation may only occur in a subset of SnRK1 complexes, precluding their detection when analysing total SnRK1 amounts. Indeed, size exclusion chromatography analyses revealed higher T-loop phosphorylation in SnRK1 complexes between 65-100KDa, which correlates with SnRK1 specific activity, suggesting that the oligomerization status can influence SnRK1 regulation (Nunes et al. 2013). Alternatively, SnRK1 may be constitutively phosphorylated and regulated by additional mechanisms.

Another difference among SNF1, AMPK and SnRK1 relates to the effect of mutations mimicking T-loop phosphorylation. In mammals, substitution of the conserved Thr with a negatively charged aspartic acid residue (T172D) results in 50% of the activity of the phosphorylated wild type kinase (Stein et al. 2000). A similar finding was reported for yeast in which the $SNF1^{T210E}$ mutant fully rescues the phenotype of the *snf1*Δ strain

(Pessina et al. 2010). In plants, despite being able to phosphorylate a synthetic peptide *in vitro* (Crozet et al. 2010), SnRK1α1^{T175D} is not able to complement the yeast *snf1*Δ mutant (Gietz et al. 1995, Glab et al. 2017), presumably due to its very low biochemical activity. Supporting this, SnRK1^{T175D} was also not capable of rescuing the Arabidopsis *snak1 snak2* double mutant (see section 1.3.1) (Glab et al. 2017).

1.3.1 Upstream kinases

Three kinases have been identified as upstream kinases of Snf1 in yeast, Sak1/Pak1, Elm1, and Tos3. Only the triple knockout mutant of these genes displays a *snf1* phenotype in response to glucose starvation, indicating that they act redundantly on SNF1 regulation (Hong et al. 2003, Sutherland et al. 2003). Nevertheless, these SNF1 upstream kinases exhibit distinct abilities to activate SNF1, depending on the β-subunit present in the SNF1 complex and the stress imposed on the cells (McCartney et al. 2005).

Homology searches with the sequences of the yeast kinases led to the discovery of mammalian LKB1 (liver kinase B1) as an AMPK upstream kinase (Hawley et al. 2003, Hong et al. 2003). LKB1 phosphorylates AMPKα in the T-loop in response to an increase in the AMP/ATP ratio and the subsequent change in AMPK conformation (Gowans et al. 2013). Biochemical analyses have further shown that LKB1 is constitutively active (Hamilton et al. 2002, Woods et al. 2003), further indicating that LKB1 activity is regulated at the substrate level. In many tissues AMPKα can also be phosphorylated on the T-loop by the Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKKb) which responds to increases in intracellular Ca^{2+} rather than to variations in the adenylate charge (Hurley et al. 2005, Woods et al. 2005, Fogarty et al. 2010). Finally, a yeast *sak1 tos3 elm1* mutant complementation screen identified the transforming growth factor-β-activated kinase (TAK1), a member of the MAPKKK kinase family, as a potential AMPK upstream kinase able to phosphorylate it *in vitro* (Momcilovic et al. 2006). However, whether TAK1 is a true upstream kinase of AMPK *in vivo* is highly controversial (Neumann et al., Int J Mol Sci 2018).

Sequence similarity with their yeast counterparts led to the identification of two Arabidopsis kinases as orthologues of Sak1/Pak1, Elm1, and Tos3, able to complement the phenotype of the yeast triple mutant (Harthill et al. 2006, Shen et al. 2006). These SnRK1 activating kinases, SnAK1 and SnAK2 (or GRIK2 and GRIK1, respectively) appear to be the only SnRK1 upstream kinases *in planta,* since mutation of both genes in the *snak1 snak2* double mutant fully abrogates SnRK1α T-loop phosphorylation (Glab et al. 2017). Earlier studies conducted *in vitro* demonstrated that both SnAK1 and 2 are able to phosphorylate the T-loop of SnRK1α leading to SnRK1 activation (Shen et al. 2009, Crozet et al. 2010). Active SnRK1, in turn, prevents its own over-activation by phosphorylating back SnAKs and thereby causing their inactivation (Crozet et al., 2010). SnAKs are mostly expressed in the apical meristems, in actively proliferating tissue such as young leaves, and in virus-infected leaf tissue (Shen et al. 2006). Similarly to the double *snrk1α1 snrk1α2* mutant (Baena-Gonzalez et al. 2007), the double *snak1 snak2* mutant is not viable in regular growth conditions (Bolle et al. 2013, Glab et al. 2017); however, it can be rescued when grown on solid medium supplied with 3% sucrose (Glab et al. 2017). Interestingly, despite its severely reduced size, the *snak1 snak2* mutant is able to grow and flower *in vitro* even though it is unfertile (Glab et al. 2017). Likewise LKB1, SnAKs appear to be constitutively active, being capable of autophosphorylation/autoactivation (Shen et al. 2009, Crozet et al. 2010).

1.3.2 Upstream phosphatases

An increasing number of studies report a central role for protein phosphatases in the control of SNF1/AMPK T-loop phosphorylation (Sanders et al. 2007, Rubenstein et al. 2008). The dephosphorylation of Snf1 in response to glucose is partly mediated by the PP1 phosphatase Glc7 that acts in a complex with the Reg1 regulatory subunit (Ludin et al. 1998, Hedbacker et al. 2008). Yeast cells lacking the Reg1 gene show a hyperphosphorylated and constitutively active Snf1, even when glucose is available in the

medium (McCartney et al. 2001), while the Glc7 mutation is lethal probably because of excessive Snf1 activity. The interaction between SNF1 and Reg1 was investigated by co-immunoprecipitation using truncated and mutant forms of Reg1, revealing that the interaction with SNF1 and Glc7 requires the same region of Reg1 and thereby excluding the formation of SNF1-Reg1-Glc7 trimers (Tabba et al. 2010). These findings prompted a model in which SNF1 T-loop dephosphorylation is regulated through a dynamic competition between the Glc7 phosphatase and the SNF1 kinase for binding to the PP1 regulatory subunit Reg1 (Tabba et al. 2010). *reg1Δ* mutants, however, are able to dephosphorylate Snf1 if glycogen synthesis is suppressed, presumably due to higher glucose accumulation under these conditions. This observation led to the identification of an additional Snf1 phosphatase (Ruiz et al., 2011), the type 2A-phosphatase Sit4 which had previously been implicated in the mitotic G1/S transition, in the target of rapamycin (TOR) pathway and in control of glycogen synthesis (Sutton et al. 1991, Di Como et al. 1996, Jablonka et al. 2006, Ruiz et al. 2011). Both Reg1 and Sit4 were shown to physically interact with Snf1 in co-immunoprecipitation experiments (Ludin et al. 1998, Ruiz et al. 2011).

Based on reports on the effect of the 2c-type phosphatases Ppm1E and Ppm1F on AMPK T-loop phosphorylation (Voss et al., 2011), the yeast ortholog Ptc1 was identified as a third SNF1 phosphatase (Ruiz et al. 2013). Ptc1 is involved in numerous processes, including MAPK pathways [e.g. high-osmolarity glycerol (HOG) and cell wall integrity (CWI) pathways], the TOR (target of rapamycin) pathway, cation homeostasis, and inheritance of cellular organelles (Arino et al. 2011). Although no evidence of direct physical interaction was provided in this study, it is still possible that the phosphatase-substrate interaction is transient and labile and difficult to detect in these approaches.

Unlike the Reg1-Glc7 and Sit4 (PP1 and PP2A phosphatases, respectively), which function as complexes, the Ptc1 phosphatase (PP2C phosphatase) acts as a monomeric enzyme. Most importantly, it can only function on Snf1 only when the latter is in complex with the β and Snf4/γ subunit (Ruiz et al. 2013). Finally, Ptc1 appears to play a secondary role in

SNF1 dephosphorylation since the Δ*ptc1* mutant shows a phenotype only in combination with the Δ*reg1* or Δ*sit4* mutations (Ruiz et al. 2013).

In animals, both PP2A and PP2C protein phosphatases have been shown to efficiently dephosphorylate AMPKα *in vitro* (Davies et al. 1995, Suter et al. 2006, Sanders et al. 2007), and for the PP2C phosphatases Ppm1E and Ppm1F, *in vivo* dephosphorylation of AMPK was also reported (Voss et al. 2011). This study describes a molecular mechanism of the action of metformin on AMPK. Metformin is a widely used drug in the treatment of type 2 diabetes which uncouples mitochondrial electron transport chain at the level of complex I (Andrzejewski et al. 2014). In addition to its effect on mitochondrial bioenergetics, metformin inhibits the phosphatase activity of Ppm1E and Ppm1F resulting into a net AMPK activation (Voss et al. 2011).
In addition, siRNA-mediated silencing of the PP1-R6 holoenzyme implicates this phosphatase in the glucose repression of AMPK in pancreatic cells (Garcia-Haro et al. 2010).

In plants, one early study showed that spinach SnRK1 can be dephosphorylated *in vitro* by human PP2A and PP2C phosphatases, but the identity of the corresponding plant phosphatase(s) was not addressed (Sugden et al. 1999). This study further showed that, similarly to animal AMPK, AMP protects SnRK1 from dephosphorylation, although the concentrations of AMP used by the authors were 10-20 fold higher than those previously used with mammalian AMPK (Davies et al. 1995, Sugden et al. 1999).
More recently, a SnRK1-phosphatase interaction was suggested from the finding that two SnRK1 β-subunits are N-myristoylated, recruiting specific SnRK1 complexes to the plasma membrane (Pierre et al. 2007). A similar myristoylation-dependent localization to the plasma membrane was later shown for the PP2C74 phosphatase (Tsugama et al. 2012), which interacts with SnRK1α1 (but not with SnRK1α2) in yeast-two-hybrid and in *in vitro* pull-down assays.

In our work, presented in Chapter II of this thesis, we provided the first proof of the *in planta* interaction between SnRK1 and PP2Ca and ABI1 (Rodrigues et al. 2013), two phosphatases of a 9-member subfamily of PP2C phosphatases (Schweighofer et al. 2004). PP2Ca and ABI1 are required for SnRK1 inactivation by sugar (Rodrigues et al. 2013). Interestingly, although basal SnRK1 activity appears unaffected in quadruple *pp2c* mutants, the glucose repression of SnRK1 appears highly compromised in the absence of these phosphatases (Rodrigues et al. 2013).

PP2Ca and ABI1, on the other hand, are well-known negative regulators of SnRK2 kinases in the ABA signaling pathway and are repressed by the ABA receptors upon binding the hormone (Ma et al. 2009)(see next section). Therefore, in our work we were able to show that by repressing SnRK1, PP2Cs allow ABA to induce SnRK1 activity, providing a molecular explanation for the extensive genetic interactions reported between ABA and sugar signaling (Rolland et al. 2006).

1.3.3 Regulation by sugars

When considering the regulation of SNF1/AMPK/SnRK1 complexes by sugars it is important to make a distinction between soluble simple sugars employed in energy production and insoluble complex carbohydrates used as storage compounds. Simple sugars like glucose modulate the activity of AMPK and SNF1 indirectly as a consequence of their effect on energy metabolism and the adenylate charge, as explained in section 1.2.3. However, in yeast, glucose has been shown to exert more direct effects on the activity of PP2A and PP1 phosphatases, inducing their posttranslational activation in a regulatory subunit (Rts1, Reg1, Shp1)-dependent manner (Castermans et al. 2012). This mechanism requires glucose phosphorylation, and, at least in the case of PP1, is likely to account for the rapid inactivation of AMPK and SNF1 in response to glucose in mammals and yeast (Garcia-Haro et al. 2010, Castermans et al. 2012).

In plants, SnRK1 is activated by several types of stress that, impairing the main energy-producing processes, photosynthesis or respiration, commonly result in cellular energy

imbalance. The fact that sugar supplementation (glucose or sucrose) prevents SnRK1 activation by different stress conditions (dark stress, hypoxia, or chemical impairment of photosynthesis) indicates that the signal behind SnRK1 activation is related to energy deprivation rather than to stress *per se* (Baena-Gonzalez et al. 2007). An increasing body of evidence shows that SnRK1 activity can be inhibited by sugar phosphates, namely glucose-6-phosphate (G6P), glucose-1-phosphate (G1P) and trehalose-6-phosphate (T6P) (Toroser et al. 2000, Zhang et al. 2009, Nunes et al. 2013, Tsai et al. 2014), providing a molecular link between SnRK1 function and energy metabolism more suitable for the sugar-based, photoautotrophic metabolism of higher plants. Glucose-mediated inhibition of SnRK1 signaling appears normal in the HEXOKINASE1 mutant *hxk1/gin2* (Baena-Gonzalez et al. 2007), suggesting that even reduced G6P levels are sufficient to block SnRK1 activity or that other sugar phosphates can compensate for this defect. Interestingly, G6P and T6P have an additive inhibitory effect, while G1P and T6P act synergistically, indicating that, despite their high structural similarities, different sugar phosphates provide similar regulation by acting at distinct sites (Nunes et al. 2013). Biochemical studies using different combinations of *in vitro* reconstituted SnRK1 trimers, as well as partly purified SnRK1 complex from plants, indicate that the inhibition of SnRK1 activity by soluble sugars is mediated by a still undefined proteinacious component present in young leaves and that can be separated from the "core" SnRK1 complex (Zhang et al. 2009, Nunes et al. 2013, Emanuelle et al. 2015). Whether the different sugars phosphates inhibit SnRK1 complex through the same unidentified factor is still unknown.
A recent work, conducted *in vitro*, shows that T6P is able to directly bind to SnRK1α and repress its kinase activity (Zhai et al. 2018). It was demonstrated that physiological concentrations of T6P are indeed able to inhibit the interaction between SnRK1α and its upstream kinase, SnAK2 (Zhai et al. 2018).

While G6P and G1P require millimolar concentrations to efficiently repress SnRK1, T6P exerts similar effects at the micromolar range (Zhang et al. 2009, Nunes et al. 2013, Zhai et al. 2018), having thus been proposed to play hormone-like functions. T6P is a disaccharide synthetized as an intermediate of trehalose synthesis. Trehalose, on the

other hand, is an ancient osmoprotectant (Crowe et al. 1992), although in plants it serves such function only in a few species like the resurrection plant, where it accumulates to extremely high concentrations during desiccation (Anselmino 1913, Crowe et al. 1992). Trehalose is produced in a two-step reaction catalyzed by two classes of enzymes: first, T6P synthase (TPS) produces T6P from G6P and UDP-glucose, and then T6P phosphatase (TPP) dephosphorylates T6P to trehalose. In plants these two gene families are over-represented compared to other organisms, suggesting an important regulatory role for trehalose metabolism in higher plants (Broeckx et al. 2016). The Arabidopsis genome encodes for 11 TPS and 10 TPP members (Leyman et al. 2001, Lunn 2007, Lunn et al. 2014). TPS proteins can further be clustered in class I and class II subfamilies (TPS1-4, and TPS5-11, respectively, in Arabidopsis) (Leyman et al. 2001, Lunn 2007), but only class I isoforms have *in vitro* TPS enzymatic activity and are able to complement the yeast *tps1 Δ* mutant (which cannot grow in medium where glucose is the only carbon source) (Blazquez et al. 1998, Zentella et al. 1999, Vogel et al. 2001, Ramon et al. 2009). Despite not being catalytically active, class II TPS proteins are likely to play a regulatory role in trehalose metabolism, (Ramon et al. 2009).

Both trehalose and especially T6P are present in very low amounts and only in the past two decades protocols were established to measure these metabolites in a few laboratories (Roessner et al. 2000, Lunn et al. 2006, Delatte et al. 2009, Carillo et al. 2013, Mata et al. 2016). These technical advances allowed the discovery of a very robust correlation between sucrose and T6P levels in Arabidopsis rosettes and other sink and sources tissues. Based on this findings, the so-called T6P-sucrose nexus model was proposed, in which T6P acts as a proxy of the sucrose status, playing insulin-like functions to maintain constant sucrose levels at the cellular and whole plant levels (Lunn et al. 2006, Lunn 2007, Lunn et al. 2014, Figueroa et al. 2016). Indeed, several studies indicate that reduced T6P levels promote sink strength and thereby the ability of sink tissues to import sucrose and grow, raising the T6P pathway as a promising target for improving crop productivity (Paul et al. 2018).

On the other hand, altered T6P levels have been increasingly associated with a wide range of developmental defects, including embryo development (Gomez et al. 2006), phase transitions (Eastmond et al. 2002, van Dijken et al. 2004) and shoot branching (Chary et al. 2008, Fichtner et al. 2017). Furthermore, mutants with increased T6P levels (due to *TPS* overexpression) show reduced sensitivity to glucose and ABA (Schluepmann et al. 2003, Avonce et al. 2004) while mutants with reduced T6P levels (due to *TPP* overexpression) show hypersensitivity (Schluepmann et al. 2003, Vandesteene et al. 2012). However, whether all the downstream effects of T6P are due to its inhibition of SnRK1 activity remains unclear and represents a major question in this field.

In addition to soluble sugars, more complex storage carbohydrates have been shown to impact SNF1/AMPK/SnRK1 activity. In the case of AMPK, the β-subunits bind glycogen *in vitro* through their glycogen binding domains (GBDs) (Polekhina et al. 2003, McBride et al. 2009). It was demonstrated that binding of glycogen to AMPKβ subunit provokes its allosteric inactivation and the extent of inactivation positively correlates with the levels of glycogen branching (McBride et al. 2009). Furthermore, the interaction between AMPKβ and glycogen allows the recruitment of the AMPK complex to the glycogen surface, inhibiting the interaction with upstream activating kinases (McBride et al. 2009, Xiao et al. 2013). Interestingly, the ability of AMPKβ to bind glycogen is prevented by *cis*-autophosphorylation on the GBD (residue Thr148) (Oligschlaeger et al. 2015), suggesting that AMPK activity controls its own sensitivity to the presence of glycogen. This mode of regulation seems to be unique to AMPKβ since the autophosphorylated residue is not conserved in the Arabidopsis or yeast orthologs (Broeckx et al. 2016). Although the yeast β subunits have been shown to bind glycogen *in vitro* (except for SIP1)(Wiatrowski et al. 2004), SNF1 activity is not affected in mutant strains in which glycogen synthesis is abolished (Momcilovic et al. 2008). Nevertheless, in the *Δreg1* mutant (with impaired Glc7 PP1 phosphatase activity), which hyperaccumulates glycogen, the SNF1 T-loop is hyperphosphorylated but normal T-loop phosphorylation can be restored by abolishing glycogen synthesis (Ruiz et al. 2011). Collective evidence suggest that the effects of

glycogen on SNF1 are not mediated by direct binding by the β-subunit but rather by an alteration of glucose metabolism resulting from glycogen accumulation (Momcilovic et al. 2008, Ruiz et al. 2011).

In Arabidopsis, an initial *in vitro* study reported a direct binding of starch to the SnRK1β2 and SnRK1βγ subunits, with a consequent inhibition of SnRK1 activity (Avila-Castaneda et al. 2014). This was further supported by the chloroplastic localization of these and the catalytic subunits (Fragoso et al. 2009, Avila-Castaneda et al. 2014). More recently, using immunogold labeling, the same group detected all subunits in the chloroplast, with SnRK1β3 and SnRK1βγ being particularly enriched in starch granules (Ruiz-Gayosso et al., 2018). Furthermore, they showed that maltose is able to bind to single SnRK1β and SnRK1β/βγ combinations and that it promotes the activity of SnRK1β3/βγ containing complexes purified at dusk. This suggests that maltose could have a positive feedback effect on starch degradation through the stimulation of SnRK1 activity at the end of the day (Ruiz-Gayosso et al., 2018). However, these results could not be confirmed by another group exploiting also different techniques (Emanuelle et al. 2015), and potential artifacts derived from protein oxidation/precipitation as well as from too high maltose concentrations have been proposed to explain these controversies (Emanuelle et al. 2015, Ruiz-Gayosso et al. 2018). Collectively, these findings seem to support previous propositions that exclude the direct binding of carbohydrates to the GBD domains of β or βγ as an important regulatory mechanism for SnRK1 complex (Broeckx et al. 2016).

2 TOR

2.1 TOR complex in yeast and mammals

TOR belongs to a family of serine/threonine protein kinases known as PIKKs (phosphoinositide 3-kinase-related kinases), which show a preference towards protein rather than lipid substrates (Abraham 2004).

In *Saccharomyces cerevisiae* TOR is encoded by two genes, *TOR1/DRR1* and *TOR2/DRR2* (Heitman et al. 1991), giving rise to two distinct protein complexes, TORC1 and TORC2, identified on the basis of their differential sensitivity to rapamycin (Loewith et al. 2002). The rapamycin-sensitive TORC1 consists of KOG1 (Kontroller Of Growth), LST8 (Lethal with Sec18 protein 8 – which is a common subunit between TORC1 and TORC2), TCO89 (Tor Complex One 89) and either TOR1 or TOR2 (Loewith et al. 2002, Reinke et al. 2004, Zinzalla et al. 2010). *TOR1* is not an essential gene in yeast, as it can be replaced by *TOR2* in the formation of TORC1 complexes. By contrast, the rapamycin-insensitive TORC2 specifically contains TOR2, which is indispensable for survival. TORC2 is composed by TOR2 bound to LST8, AVO1, and AVO3 (Adheres Voraciously) (Loewith et al. 2002, Reinke et al. 2004). AVO3 harbors a domain that prevents the rapamycin inhibition of TORC2, and deletion of such domain was shown to sensitize TORC2 to this drug (Gaubitz et al. 2015). In mammals, a single *mTOR* gene codifies for the catalytic subunit of two distinct protein complexes, known as mTOR complex 1 (mTORC1) and 2 (mTORC2). mTORC1 is composed by three core components: mTOR, Raptor (regulatory protein of mTOR, the mammalian ortholog of yeast Kog1) and mLST8 (also known as GβL) (Hara et al. 2002, Kim et al. 2002, Kim et al. 2003, Zinzalla et al. 2010). Raptor is required for the correct subcellular localization of mTORC1 and it allows the recruitment of substrates by recognizing the TOR signaling (TOS) motif present in many mTORC1 targets (Nojima et al. 2003). mLST8, instead, interacts with the mTOR catalytic domain, stabilizing the kinase activation loop (Yang et al. 2013). However, genetic studies suggest that it is not essential for some mTORC1 functions (Guertin et al. 2006). In mTORC2, Raptor is substituted by

Rictor (rapamycin insensitive companion of mTOR), which is the discriminant subunit to discern between mTOC1 and mTORC2 (Sarbassov et al. 2004).

TORC1 primarily controls temporal aspects of cell growth and anabolic metabolism, including the activation of lipid and protein synthesis, ribosome biogenesis, mitochondrial metabolism and inhibition of autophagy (Wullschleger et al. 2006, Wang et al. 2009). TORC2, in turn, regulates spatial aspects of growth, such as cytoskeletal organization and cellular polarization (Jacinto et al. 2004, Roelants et al. 2017). However, the functional distinction between the two TOR complexes is not as stringent as initially proposed. Indeed, the emerging picture regarding TOR and cell growth regulation indicates a higher level of complexity given that TORC1 interacts also with cytoskeletal elements, while TORC2 impacting processes like transcription, translation and cell cycle progression, initially defined as being exclusively under TORC1 control (Gonzalez et al. 2017).

2.1.1 The rapamycin dilemma

Biochemical studies identified the direct target of rapamycin as the peptidyl-prolyl-isomerase FKBP12, which becomes competent to inhibit mTOR upon rapamycin binding (Brown et al. 1994, Sabatini et al. 1994). It should be noted that in the absence of rapamycin FKBP12 does not appear to have a role in TOR signaling and its gene disruption does not inhibit growth, which initially surprised the researchers. Later studies demonstrated that rapamycin hijacks or corrupts FKBP12 to interact with TOR. Structural analyses suggest that the interaction between the rapamycin-FKBP12 complex and mTOR narrows the catalytic cleft, preventing substrate phosphorylation through steric repression of the TOR kinase or interference with the binding sites of essential TOR substrates (Yang et al. 2013, Baretic et al. 2014). Although this toxin has been speculated to mimic an endogenous metabolite that regulates TOR *via* FKBP, there is no evidence supporting the existence of such compound or such a mode of TOR regulation (Loewith et

al. 2011). Nevertheless, rapamycin sensitivity has been widely employed for investigating TOR functions allowing the identification of TORC as a central regulator of metabolism.

2.2 TOR complex in plants

To date, only TORC1 complex components have been identified in photosynthetic organisms (Henriques et al. 2014, Martins et al. 2019).

The Arabidopsis genome contains a single *TOR* gene, two genes for LST8 (*LST8-1* and *LST8-2*) and two genes for RAPTOR (*RAPTOR1A* and *RAPTOR1B*) (Menand et al. 2002, Deprost et al. 2005). RICTOR appears to be absent in higher plants (Xiong et al. 2014) suggesting that, despite the essential nature of TORC2 in animals and fungi, higher plants only possess a TORC1 complex.

As described for other systems, *TOR* is an essential gene and the Arabidopsis *tor* null mutant is embryo lethal, with embryos arresting at the 16 to 32-cells stage (Menand et al. 2002, Ren et al. 2011). To circumvent embryo lethality and elucidate the function of TOR in plants a number of tools have been developed such as *TOR* RNA interference (*TOR* RNAi) and *TOR* artificial microRNA (amiR-*TOR*) (Deprost et al. 2007, Xiong et al. 2012, Caldana et al. 2013). Using gain- and partial loss-of-function mutants, a correlation was established between the levels of *TOR* expression and polysome abundance (as a read out of the translation rate), plant size, and seed yield (Deprost et al. 2007). Furthermore, ethanol-inducible *TOR* RNAi lines reveal that, when *TOR* is silenced, genetic and metabolic senescence markers become upregulated, and autophagy undergoes constitutive activation resulting in plant growth arrest (Deprost et al. 2007, Liu et al. 2010).

The RAPTOR1A and RAPTOR1B proteins share around 80% homology and, although both genes are actively transcribed throughout development, only the *raptor1b* mutant has a clear phenotype, displaying delayed development (Anderson et al. 2005). Quantification of its growth dynamics, however, reveals that this developmental delay is not linear, as

would be expected from a continuous growth repression, but that it rather occurs at distinct developmental stages, namely during germination, during the transition from juvenile to the adult vegetative stage, and during flowering and subsequent silique ripening (Salem et al. 2018). In contrast to the *tor* mutant, the *raptor1a raptor1b* double mutant produces viable embryos but undergoes developmental arrest post-embryonically (Anderson et al. 2005). Assuming that the TOR kinase is always part of a protein complex, these observations suggest the existence of an elusive plant TOR complex independent from RAPTOR*,* although no TORC2-specific components have been identified in plants.

The Arabidopsis genome contains two genes encoding LST8/GβL orthologs, *LST8-1* ad *LST8-2,* but only *LST8-1* seems to be significantly expressed. In short day conditions *lst8-1* mutants show mild growth defects, displaying mostly a slight reduction in rosette size and a late flowering phenotype (Moreau et al. 2012). In long days, however, the growth of the *lst8-1* mutant is highly compromised, showing smaller rosettes and a marked developmental delay. At the flowering stage, they become bushy, developing multiple stems and very small siliques with a high rate of aborted seeds (Moreau et al. 2012).

The growth phenotype of genetically or chemically repressed TOR plants, as well as TOR complex components mutants, LST8 and RAPTOR1, is associated with hyperaccumulation of starch (Deprost et al. 2007, Moreau et al. 2012, Caldana et al. 2013, Salem et al. 2018). This phenotype is in agreement with the observed accumulation of glycogen in yeast and mammals (Laplante et al. 2012). Network analyses of metabolite profiles in a large population of Arabidopsis accessions revealed that starch levels are inversely correlated with biomass production (Sulpice et al. 2009). Similarly to animals and fungi, the activation of TOR in plants leads to large changes in metabolism promoting the production of simple metabolites for immediate growth while inhibiting the synthesis of complex compounds used as storage to sustain growth in longer-term (Rexin et al. 2015). In addition to starch, carbon can be stored as lipids, especially triacylglycerides (TAGs) which are also accumulating in TOR mutants plants and algae (see section 2.3.3.2). Taken together, the findings on starch and lipids accumulation in TOR mutants, corroborate the

idea that accumulation of storage compounds is associated with TOR-mediated repression of growth.

2.2.1 Usage of rapamycin in plant research

In the unicellular algae *Chlamydomonas reinhardtii,* FKBP12 is largely similar to its human and yeast counterparts, allowing the repression of TOR activity with moderate rapamycin concentrations (100-500 nM) that inhibit also yeast growth (Crespo et al. 2005). In higher plants, on the other hand, TOR is relatively insensitive to rapamycin, a fact that has significantly delayed studies on its function (Menand et al. 2002). The rapamycin insensitivity is due to differences in FKBP12 residues that are necessary for the formation of the FKBP12-rapamycin-TOR ternary complex and, accordingly, heterologous expression of either human (Mahfouz et al. 2006) or yeast (Sormani et al. 2007) FKBP12 in Arabidopsis confers rapamycin sensitivity. However, it has been reported that repeated treatment of Arabidopsis seedlings with high rapamycin concentrations (1-10 µM) can reduce growth as well as phosphorylation of a downstream target and that growth inhibition is dependent on endogenous FKBP12 (Xiong et al. 2012). More recently, a new class of mTOR inhibitors has been developed that act in a FKBP12-independent manner (e.g. torin1, torin2, AZD-8055). Known as second generation TOR inhibitors, this class of compounds is able to bind the ATP binding pocket of the TOR kinase, thus competing with ATP and inhibiting both TORC1 and TORC2 activities (Zhang et al. 2011). As expected, these chemicals are potent inhibitors of growth also in plants (Montane et al. 2013, Li et al. 2015), although their specificity for TOR kinase compared to other related plant kinases has not yet been fully characterized (Montane et al. 2013). The availability of these chemical inhibitors in combination with highly efficient genetic tools (see below) has contributed to a much more rapid growth of the plant TOR field as compared to that of SnRK1 signaling.

2.3 TOR functions in plants

In accordance with other organisms, in plants TOR promotes major anabolic processes including protein translation and nucleic acid synthesis. Moreover, in the case of plants, TOR is implicated in the regulation of nitrogen metabolism and carbon/nitrogen ratio.

2.3.1 Translation

One major function of TOR is the induction of protein synthesis. In mammals this is accomplished through the phosphorylation-mediated inhibition of 4EBP (eIF4E-binding protein) proteins that repress translation initiation and through the phosphorylation-mediated activation of ribosomal protein 6 kinase (S6K1 or p70S6K), which in turn phosphorylates ribosomal protein 6 (RPS6) (Holz et al. 2005, Mahfouz et al. 2006, Wullschleger et al. 2006, Sonenberg et al. 2009). S6K is a widely studied effector of mTORC1 activation, orchestrating cell physiology by regulating fundamental cellular processes including translation, transcription, lipid synthesis, cell metabolism and growth (Topisirovic et al. 2011, Magnuson et al. 2012). Similarly to mammals, plants possess two genes encoding S6K (*S6K1* and *S6K2*) (Mizoguchi et al. 1995, Lee-Fruman et al. 1999). The TOR consensus phosphorylation motif (FLGFTYVAP) of human S6K (Nojima et al. 2003) is fully conserved in the Arabidopsis orthologs, S6K1 and S6K2 (corresponding to T449 in S6K1), and responds to inhibition of TOR activity by rapamycin (Xiong et al. 2012) and by torin and AZD-8055 (Wang et al., 2017), making it a robust biochemical marker of TOR activity also in plants (Xiong et al. 2012, Schepetilnikov et al. 2013, Kravchenko et al. 2015, Dobrenel et al. 2016, Li et al. 2017).

Plants appear to lack 4EBP proteins and no evidence that TOR regulates translation initiation has been reported (Rexin et al. 2015). Instead it controls translation of mRNAs harboring uORFs (upstream open reading frames) in the 5'UTR by promoting translation reinitiation (Rexin et al. 2015). Depending on the length of uORFs (and consequently on

the time that ribosomes take to translate them) and the distance between these uORF and the main one, protein expression can be reduced by 30-80%. This occurs without changes in mRNA abundance by causing the dissociation of the initiation complex during the translation of these ORFs (Calvo et al. 2009). uORFs are present in about 30% of Arabidopsis mRNAs (Zhou et al. 2010) regulating fundamental processes related to metabolism and development (Rosado et al. 2012, von Arnim et al. 2014, Zhou et al. 2014).

Mechanistic insight into how TOR modulates translation came from studies showing TOR activation by auxin and viral infection and phosphorylation of eIF3H (eukaryotic initiation factor 3H) (Schepetilnikov et al. 2011, Rosado et al. 2012, Schepetilnikov et al. 2013)(see section 2.4.2). In response to auxin, TOR associates with polysomes, phosphorylating S6K and causing its activation and dissociation from polysomes (Bogre et al. 2013, Schepetilnikov et al. 2013). Activated S6K, in turn, phosphorylates eIF3H stabilizing the ribosome-mRNA association when transiting on uORF STOP codons, allowing polysome loading and translation reinitiation of the main ORF in uORF-containing mRNAs (Bogre et al. 2013, Schepetilnikov et al. 2013). The role of TOR and eIF3 phosphorylation in auxin signaling was further validated by the observation that *TOR* RNAi lines and *eif3* mutants display defective auxin responses like gravitropism (Schepetilnikov et al. 2013).

2.3.2 Transcription

Activation of TOR signaling in response to glucose results in a rapid transcriptional reprogramming in which genes related to anabolic processes (e.g. RNA transcription and amino acid, lipid, protein and DNA synthesis) are upregulated, while genes involved in catabolic processes (degradation of proteins, amino acids, lipids and autophagy) are downregulated (Xiong et al. 2013, Xiong et al. 2013). Noteworthy, TOR regulates transcription in plants through a novel mechanism not described in other organisms (Laplante et al. 2012, Martins et al. 2019). TOR phosphorylates the N-terminal region of

the E2Fa transcription factor, promoting its activity and consequent activation of S-phase genes, among others (Xiong et al. 2013, Xiong et al. 2013). E2Fs are known targets of the CYCLIN (CYC)-DEPENDENT KINASE (CDK)/RETINOBLASTOMA-RELATED PROTEIN (RBR) axis that regulates cell cycle progression (De Veylder et al. 2007), but the effect of TOR on E2Fa appears to be independent of these factors (Xiong et al. 2013, Xiong et al. 2014).

2.3.3 Other TOR functions in plants

2.3.3.1 Nitrogen metabolism

In addition to the functions described in the previous section TOR appears to be important for the regulation of nitrogen metabolism as well as for establishing a nitrogen and carbon balance.

Nitrogen is an essential nutrient for plant survival that is adsorbed from the soil in the form of nitrate (Xu et al. 2012). In order to be available for plant metabolism, nitrate is subjected to two consecutive steps of reduction and incorporated into glutamate giving rise to glutamine (which contains one amide as functional group) (Xu et al. 2012). Another important source of nitrogen is nitrogen re-assimilation from protein degradation, achieved through the cytosolic glutamine synthetase (GS1) and glutamate dehydrogenase (GDH) activity, especially during senescence (Pageau et al. 2006). Nitrogen assimilation and the accumulation of glutamine are increased in plant lines with compromised TOR activity either as the consequence of conditional silencing of TOR, or because of knock out mutation in components of TOR complex such as RAPTOR1B or LST8 (Deprost et al. 2007, Moreau et al. 2012, Salem et al. 2018). The effect of TOR on nitrogen metabolism is, at least partly, mediated by the plant TOR effector TAP46 (2A PHOSPHATASE ASSOCIATED PROTEIN OF 46 KDa) (Ahn et al. 2011). Genetic evidence suggests that silencing of *TAP46* affects nitrogen metabolism by inhibiting nitrogen assimilation and favoring nitrogen remobilization. Similarly to TORC down-regulated lines, TAP46 RNAi

Arabidopsis plants present a lower nitrate reduction activity with concomitant upregulation of genes encoding GS1 and GDH for nitrogen re-assimilation (Ahn et al. 2011, Ahn et al. 2015). By contrast, lines with inducible TAP46 overexpression present an overall enhanced plant growth associated with increased nitrogen assimilation and slightly reduced GS activity (Ahn et al. 2015).

All these results partly contrast with the observations from estradiol-inducible amiR-*TOR* lines in which primary metabolites were quantified by GC-TOF MS (gas chromatography coupled with mass spectrometry) (Caldana et al. 2013). In this study the levels of central intermediates of nitrogen metabolism such as glutamate and glutamine, were not up-regulated. On the contrary, a significant decrease of glutamine and other nitrogen-containing intermediates (arginine, ornithine and citrulline) was detected (Caldana et al. 2013). This discrepancy between experiments has been attributed to differences in the carbon supply, thereby linking the TOR-dependent regulation of nitrogen metabolism (assimilation and/or recycling) to the carbon availability in the growth medium. In particular, TORC repression seems to promote nitrogen assimilation under normal carbon conditions (Ahn et al. 2011, Moreau et al. 2012) and to repress nitrogen incorporation into amino acids under increased carbon availability (e.g. sucrose surplus in the growth medium) (Caldana et al. 2013).

2.3.3.2 Lipids accumulation

In Arabidopsis, knocking down the *TOR* gene or knocking out other TOR complex components results in over-accumulation of TAGs, especially poly-unsaturated fatty acids (Moreau et al. 2012, Caldana et al. 2013, Salem et al. 2017). Similar responses have been reported in green and red algae (Lee et al. 2013, Imamura et al. 2015). Taken together, the findings in these two unicellular photosynthetic organisms indicate that TOR signaling on TAGs accumulation originated before the differentiation of the viridiplantae lineage.

A different study on rice, in which TOR is chemically inactivated (with AZD-8055, or torin2), reports that TOR and S6K repression is associated with depletion of specific galactolipids (Sun et al. 2016). Galactolipids are major constituents of thylakoids membranes, therefore TOR-dependent defects in lipogenesis of this class of compounds result in pale leaves and chloroplast morphology aberrations (Sun et al. 2016).

2.4 Regulation of TOR activity

TOR exerts a pivotal role in sensing and integrating nutrient and energy availability with environmental signals in order to coordinate growth and development. Several studies extensively demonstrated that TOR is activated by nutrients and hormones, but is inactivated by energy depletion, nutrient starvation and various other stresses in yeast, animals and plants (Rexin et al. 2015, Xiong et al. 2015, Gonzalez et al. 2017, Saxton et al. 2017). In mammals, TOR activity is mainly linked with amino acid sensing and insulin (or other growth factors) (Wullschleger et al. 2006, Bar-Peled et al. 2014), but in plants and yeast an effect of amino acids on TOR activation has not been consistently established (Dann et al. 2006, Xiong et al. 2013, Xiong et al. 2015), possibly because of their ability to synthetize all 20 aminoacids. In the case of yeast, a recent study employing several auxotroph strains suggests an alternative TOR regulating system by which specific tRNAs can inhibit TOR activity (Kamada 2017). Whether a similar mechanism operates also in plants is not currently known.

2.4.1 Regulation by glucose

One major regulator of TOR activity in plants is glucose (Xiong et al. 2012). When Arabidopsis seedlings are grown in low light conditions and sugar-free liquid medium they initiate photomorphogenesis, but arrest growth 3 days after germination (DAG) due to the exhaustion of seed reserves and their incomplete photosynthetic capacity. Several

lines of evidence demonstrate that the root apical meristem enters a mitotically quiescent state as a consequence of low TOR activity and that this state can be reversed by the provision of glucose or sucrose, but not other sugars (i.e. fructose, xylose and galactose) or hormones (i.e. auxin, cytokinin, brassinosteroid, or gibberellin) (Xiong et al. 2013). Furthermore, these sugars need to be metabolized through glycolysis and mitochondrial respiration, suggesting that glucose is not directly involved in TOR re-activation (Xiong et al. 2013).

2.4.2 TOR regulation by hormones

In addition to nutrients, TOR is regulated by hormones (Dong et al. 2015) such as growth-promoting auxin. Auxin activates TOR to promote translation reinitiation of uORF-containing auxin-regulated mRNAs (Schepetilnikov et al. 2013). Auxin is required to activate TOR signaling both in shoot and root apical meristems (SAM and RAM) (Li et al. 2017). In the case of the RAM glucose supply is sufficient to activate TOR signaling, whereas in the SAM, light is additionally required, likely to maintain auxin levels sufficiently high (Li et al. 2017). The activation of TOR by auxin is dependent on the interaction between TOR and the small GTPase Rho-related protein 2 (ROP2) (Schepetilnikov et al. 2017). ROP2 is however not involved in the activation of TOR by glucose, indicating that TOR senses sugars through another upstream regulator (Li et al. 2017).

Increasing connections have also been established between TOR and ABA signaling. In Arabidopsis, TOR complex mutants (*raptor* and *lst8*) and seedlings treated with TOR inhibitors are more sensitive to ABA (Kravchenko et al. 2015, Salem et al. 2017), whereas in rice TOR overexpressors display ABA insensitivity during germination (Bakshi et al. 2017). However, other studies report that plants overexpressing TOR are more resistant to ABA-related stresses like osmotic stress (developing longer roots), whereas silencing *TOR* had the opposite effect (Deprost et al. 2007). TOR overexpression in rice was also

associated with improved growth under drought (Bakshi et al. 2017), whereas Arabidopsis *TOR* RNAi lines were more sensitive to cold stress (Dong et al. 2018). These conflicting results could potentially be explained by differences in the severity and duration of the stress treatments, as the ability to promote root growth and/or synthesize osmoprotective sugars/amino acids *via* TOR activation is likely to be beneficial for coping with certain stresses. TOR is indirectly linked to ABA *via* BIN2, a GSK3-like kinase that negatively regulates brassinosteroid signaling. BIN2 is phosphorylated and repressed by S6K2 in a TOR-dependent manner to induce photoautotrophic growth in Arabidopsis seedlings (Xiong et al. 2017), presumably through repression of ABA signaling. This effect is in agreement with the previously identified role of BIN2 as a positive regulator of the ABA pathway through direct ABA-triggered phosphorylation and activation of SnRK2 kinases (Cai et al. 2014). A more direct involvement of TOR in ABA signaling was described in a recent study in which TOR was shown to phosphorylate the ABA receptors in non-stressed plants to prevent leaky ABA signaling. Phosphorylation of the receptors disrupts their association with ABA and with the PP2C phosphatase repressors, leading to inactivation of SnRK2 kinases, the main positive effectors of the ABA pathway (Wang et al. 2018)(see section 3.2). In the same study S6K phosphorylation was used as readout to show that ABA inhibits TOR signaling, potentially through a decreased association of RAPTOR with TOR in the presence of the hormone (Wang et al. 2018). Furthermore, TOR repression by ABA required SnRK2 kinases, which interacted with RAPTOR in yeast-two-hybrid and were able to phosphorylate RAPTOR *in vitro*. Altogether this led authors to conclude that SnRK2s repress TOR in response to ABA to downregulate growth during stress (Wang et al. 2018). However, results presented in Chapter III of this thesis argue against this model and suggest instead that SnRK2s are required to form ABA-responsive repressor complexes of SnRK1 and that SnRK1, and not SnRK2s, interacts with TOR to repress TOR signaling in the presence of ABA.

Finally, chemical inhibition of TOR with AZD-8055 blocks early seedling development but mutants of the ABA-dependent transcription factor ABI4 are insensitive, developing fully expanded green cotyledons in the presence of the inhibitor (Li et al. 2015). This indicates

that TOR signaling controls the heterotrophy to autotrophy transition by acting directly or indirectly on ABI4.

Besides affecting ABA signaling, TOR has also an effect on ABA accumulation. Reduced TOR activity results in lower ABA accumulation by reducing the expression of genes involved in ABA synthesis while increasing those of ABA catabolism (Kravchenko et al. 2015). Interestingly, phenotypical analysis of Arabidopsis *raptor* mutants reveals an ABA hyperaccumulation in mature seeds (Salem et al. 2017) while, according with previous results, ABA levels decline during vegetative growth (Salem et al. 2018) indicating that the developmental stage has a big impact in the hormone accumulation in this mutant.

2.5 TOR/SnRK1 regulation

In mammals mTOR activity is inhibited by AMPK through direct phosphorylation of RAPTOR under energy starvation conditions (Gwinn et al. 2008). Conversely, it was found that in the hypothalamus S6K prevents AMPK activation through Ser491 phosphorylation when nutrients are abundant (Dagon et al. 2012), thereby establishing an inhibitory loop based on reciprocal phosphorylation of TOR and AMPK complexes. mTOR is additionally inhibited by AMPK in an indirect manner, through the phosphorylation of Tuberous Sclerosis Complex 2 (TSC2) which, in turn, is promoted by LKB1-dependent activation of AMPK (Inoki et al. 2003, Shaw et al. 2004). Finally, Snf1 T-loop phosphorylation results to increase upon blocking of TOR activity with rapamycin suggesting an inhibitory role of TOR in SNF1 regulation (Orlova et al. 2006).

Despite the poor conservation of some specific components of the AMPK-mTOR axis, and the marked difference in the energy metabolism between mammals and plants, the overall organization and functional relationship of these two pathways is evolutionarily conserved (Roustan et al. 2016). In accordance with this, SnRK1 and TOR manipulation results in largely opposite transcriptional responses regarding, in particular, regulation of

anabolic and catabolic processes (Baena-Gonzalez et al. 2007, Caldana et al. 2013, Xiong et al. 2013, Xiong et al. 2013).

To investigate the overall role of SnRK1 and to identify potential SnRK1 targets, a phospho-proteomic approach was undertaken to compare wild type and a SnRK1α knockdown (KD) (inducible amiRNA$^{SnRK1\alpha2}$ in a *snrk1α1* background) in response to energy deficit (Nukarinen et al. 2016). In response to night extension, SnRK1α KD plants display higher phosphorylation levels of TOR targets (e.g. RPS6 and translation initiation factor eIF5A) compared with wild type (Nukarinen et al. 2016). Moreover, SnRK1α and RAPTOR interact in the cytosol in Bimolecular Fluorescent Complementation (BiFC) assays and SnRK1α is able to phosphorylate RAPTOR1b *in vitro* (Nukarinen et al. 2016), altogether suggesting that, similarly to mammals, SnRK1 can inhibit TOR activity by phosphorylating RAPTOR. The interaction between SnRK1α and RAPTOR was further confirmed in yeast-two-hybrid experiments, and appears to be facilitated by the scaffold protein DUF581-19/MARD1 (Nietzsche et al. 2016).

Unlike mammals, direct phosphorylation of SnRK1 by S6K, or by TOR itself, has not been reported for plants. However, TOR and SnRK1 signaling are connected at multiple levels downstream of the two kinase complexes, co-regulating fundamental cellular functions in stress and in optimal conditions. One example of such co-regulated process is autophagy. Stress-induced autophagy is an evolutionarily conserved process by which cells degrade damaged, or unnecessary, cytoplasmic constituents and organelles, to recycle fundamental materials into new building blocks or for energy production necessary to cope with stress (Ustun et al. 2017, Avin-Wittenberg 2019). In plants, likewise in yeast and mammals, SnRK1 and TOR regulate autophagy in a positive and negative fashion, respectively (Pu et al. 2017, Soto-Burgos et al. 2018). Overexpression of SnRK1 increases the basal level of autophagy (Chen et al. 2017, Soto-Burgos et al. 2017), which is similar to the effects of chemical or genetic inhibition of TOR activity (Liu et al. 2010, Pu et al. 2017, Salem et al. 2018). Simultaneous manipulation of both SnRK1 and TOR activities revealed that SnRK1 is upstream of TOR in the regulation of autophagy (Soto-Burgos et al. 2017):

autophagy is not induced when both SnRK1 and TOR activities are increased, while constitutive autophagy occurs when both SnRK1 and TOR activities are diminished (Soto-Burgos et al. 2017). Finally, SnRK1 is able to induce autophagy in response to different stress conditions such as energy starvation, osmotic and salt stress, oxidative and ER stress, and TOR overexpression generally inhibits stress-induced autophagy (Soto-Burgos et al. 2017). Interestingly, TOR overexpression does not prevent the autophagy induction by oxidative and ER stress suggesting that in response to specific stimuli SnRK1 can induce autophagy in a TOR-independent manner (Pu et al. 2017, Soto-Burgos et al. 2017).

Given its role in degradation and recycling of cellular components, autophagy can potentially regulate several cellular processes by degrading positive and negative regulators. For example, BZR1, a transcription factor acting as positive regulator of brassinosteroid signaling is degraded during carbon starvation in an autophagy-dependent manner while it is stabilized in nutrient rich condition by TOR-dependent autophagy inhibition (Zhang et al. 2016).

3 ABA

Abscisic acid (ABA) is a sesquiterpene that acts as a phytohormone, playing crucial roles in plant growth and development and in abiotic stress responses. It has been found in various species of the Tracheophyta group, but also in more ancient and divergent groups of the plant lineage such as Bryophyta (mosses). ABA levels increase in response to water stress in all land plants from bryophytes to angiosperms (Takezawa et al. 2011).

Because of its antiquity and its prominent role during water deficit, the evolution of the ABA pathway is considered a milestone for invasion and adaptation to soil of land plant ancestors (Hauser et al. 2011, Sakata Y. et al. 2014, Shinozawa et al. 2019). Our knowledge on ABA metabolism and function is particularly extensive in angiosperms. In Arabidopsis nearly all the steps of ABA synthesis and degradation have been elucidated, as well as many aspects of ABA signaling at the molecular level (Vishwakarma et al. 2017, Ma et al. 2018).

ABA is an important signal in the adaptation to numerous abiotic and biotic stresses. Drought, osmotic and high salinity stress are perceived at least partly through ABA signaling (Raghavendra et al. 2010); ABA also mediates temperature-triggered stresses (response to cold and heat) as well as high light signaling and responses to heavy metals (Fediuc et al. 2005, Galvez-Valdivieso et al. 2009, Raghavendra et al. 2010, Shi Y. et al. 2014, Huang et al. 2016). ABA plays also an important role in plant-microbe interactions, both with regard to pathogens (Sanchez-Vallet et al. 2012, Sivakumaran et al. 2016) and symbionts (Herrera-Medina et al. 2007, Stec et al. 2016). Emerging evidence indicates several layers of interactions between ABA and pathogen-related phytohormones, namely salicylic, jasmonic acid, and ethylene (Shigenaga et al. 2016, Verma et al. 2016).

The short-term responses mediated by ABA represent a first line of protection against stress, aiming at restoring the plant water potential through modulation of stomata

conductance and the production of osmo-compatible solutes. When the stress situation is prolonged, developmental plasticity provides the most important mechanism of adaptation, allowing tolerance in the long-term. ABA plays a pivotal role in environmentally-regulated developmental processes, especially in the modulation of germination and seedling establishment, root architecture, shoot growth and branching, and root-shoot carbon partitioning (Gonzalez-Grandio et al. 2014, Harris 2015, McAdam et al. 2016).

Although ABA signaling has been mostly studied in the context of stress responses, ABA plays important roles in development also in non-stress conditions, affecting a wide range of processes from seed maturation and germination to flowering and senescence (Yan et al. 2017, Zhao et al. 2017, Shu et al. 2018). Moreover, ABA acts in concert with other hormones, metabolites and second messengers to regulate the growth rate of important organs like roots, the patterning of stomata in leaves, and fruit ripening (Leng et al. 2014, Serna 2014, Harris 2015).

3.1 ABA homeostasis: biosynthesis, degradation, glucosylation and transport.

ABA levels are regulated by an intricate network involving ABA biosynthesis, degradation, glucosylation and transport. The control of these processes enables plants to dynamically modify ABA levels at specific developmental stages, as well as in response to biotic and abiotic stresses (Nambara et al. 2005).

In higher plants, ABA synthesis starts in the chloroplast from intermediates of the xanthophyll cycle and is completed in the cytosol (Schwartz et al. 2003, Nambara et al. 2005). Xanthophylls are a class of carotenoid compounds that limit reactive oxygen species (ROS) formation from high light irradiation (Latowski et al. 2011). The product of the *ABA1* (zeaxanthin epoxidase, ZEP) gene catalyzes a two-step reaction leading to the

formation of violaxanthin which, in turn, is converted to neoxanthin by ABA4. Both 9-cis-neoxanthin and 9-cis-violaxanthin can be cleaved by 9-cis-epoxycarotenoid dioxygenase (NCED) to produce xanthoxin that is exported outside the plastid. The reaction catalyzed by NCED enzymes (9 members in Arabidopsis) is considered the rate-limiting reaction in ABA synthesis (Iuchi et al. 2001). Once in the cytosol, xanthoxin is converted to ABA through two consecutive reactions, first abscisic aldehyde is produced by an alcohol dehydrogenase (ABA2) and finally the aldehyde is converted to abscisic acid by abscisic aldehyde oxidase (AAO3).

ABA degradation is achieved by irreversible methyl group hydroxylation mediated by a class of cytochrome P450 monooxygenases (CYP707), which catalyzes the formation of phaseic acid (PA) that is further converted into dihydrophaseic acid (DPA) (Kushiro et al. 2004). Both PA and DPA have low or no biological activity, respectively (Zhou et al. 2004).

ABA homeostasis is also regulated by glucose conjugation, which results in the formation of ABA glucosyl ester (ABA-GE) that is biologically inactive (Priest et al. 2006). Compared to ABA hydroxylation, ABA glucosylation is a reversible esterification of the phytohormone, catalyzed in Arabidopsis by three redundant uridine diphosphate glucosyltransferases (UGT71B6/7/8). Arabidopsis mutants silenced for the three genes (*ugt71B6/7/8* RNAi) display multiple growth defects, enhanced resistance to osmotic stress and hypersensitivity to exogenous ABA (Dong et al. 2014).

Besides affecting its activity, glucosylation changes ABA intracellular localization (Dietz et al. 2000, Kim et al. 2013). ABA-GE represents a transported form that can be stored in the vacuole and endoplasmic reticulum where it can be converted into active ABA in a one-step glucosidase reaction. In Arabidopsis, three genes responsible for ABA-GE glucosidation were identified, the product of *AtBG1* is localized in the ER while AtBG2 and BGLU10 are found in vacuoles (Xu et al. 2012). Importantly, the ABA glucosidase function rapidly increases in response to water stress (Lee et al. 2006, Xu et al. 2012), indicating that ABA reactivation from intracellular stores is an important strategy to rapidly cope with stress conditions.

The main organs that synthesize ABA are roots and leaves (Shi Y. et al. 2014). Several lines of evidence indicate that ABA biosynthesis enzymes (e.g., NCED3, ABA2, AAO3) are mainly localized in the vascular system of leaves (Koiwai et al. 2004, Endo et al. 2008, Kuromori et al. 2014), suggesting that ABA translocates across cell layers to reach guard cells and modulate their conductivity. Moreover, ABA is a weak acid (pKa 4.7) that primarily exists in a deprotonated state (anionic form) at the physiological pH of the cytosol (7.2 -7.4), implying that active transport mechanisms are required to translocate ABA across cell membranes. In Arabidopsis, four ABA transporters localized in the plasma membrane have been identified, ABCG25, ABCG40, NPF4.6/NRT1.2/AIT1 and DTX50 (Kuromori et al. 2018). ABCG25 and ABCG40 belong to the ABC class transporters, and function as ABA exporter and importer, respectively (Kang et al. 2010, Kuromori et al. 2010). ABCG25 is expressed in the vasculature of leaves and roots (Kuromori et al. 2010), whereas ABCG40 has a broader expression pattern, with an enrichment in guard cells (Kuromori et al. 2010). NPF4.6/NRT1.2/AIT1 was initially identified as a nitrate transporter; however it presents a much higher affinity for ABA than nitrate (ABA K_m = 5 µM compared to nitrate K_m = 5.9 mM) (Kanno et al. 2012). DTX50 belongs to the multidrug and toxin efflux class (MATE) and the corresponding *dtx50* mutant exhibits growth defects possibly due to higher sensitivity to ABA or higher ABA levels (Zhang et al. 2014)

A recent report showed that ABA synthesis and subsequent stomata closure is induced in leaves by CLE25, a small peptide from the CLAVATA3 family synthesized in roots in response to water shortage that is transported to the leaves inducing ABA production (Takahashi et al. 2018).

3.2 The “core” ABA signaling

An ABA core model started to emerge following the ground-breaking discovery of the ABA receptors PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) (hereafter referred as PYR/PYLs) (Cutler et al.

2010). In the absence of ABA, the positive effectors of the pathway, subclass III SnRK2 kinases, are kept inactive by clade A PP2C phosphatases (Ma et al. 2009, Park et al. 2009, Umezawa et al. 2009), which physically interact with SnRK2 kinases and dephosphorylate a key serine residue in the kinase activation loop (Belin et al. 2006, Boudsocq et al. 2007, Vlad et al. 2009). Structural evidence suggests that PP2C phosphatases repress SnRK2 activity by two complementary mechanisms, by direct dephosphorylation of the T-loop and by physically blocking the access of substrates to the kinase active site (Soon et al. 2012). In the presence of ABA, the hormone binds to its receptors, causing a conformational change that allows the formation of a ternary complex with PP2C (PYR/PYLs-ABA-PP2C) and the release of SnRK2s (Melcher et al. 2009, Yin et al. 2009, Zhang et al. 2012). The structures of ABA receptors in a free state, bound to ABA (PYR/PYLs-ABA) and PYR/PYLs-ABA-PP2C complex were solved at high resolution, revealing a "gate-latch-lock" mechanism by which PYR/PYLs can mediate ABA-dependent inhibition of PP2C (Melcher et al. 2009, Melcher et al. 2010). Once bound to ABA two highly conserved loops in the PYR/PYLs come into proximity, acting as a gate and latch that structurally mimics the SnRK2-PP2C interface allowing the displacement (and activation) of free SnRK2 kinases (Soon et al. 2012).

The Arabidopsis genome codifies for 10 SnRK2 protein kinases, among which those of subclass III (SnRK2.2, SnRK2.3 and SnRK2.6) are strongly induced by ABA (Nakashima et al. 2009, Kulik et al. 2011). In the absence of PP2Cs the SnRK2 kinases are in a T-loop phosphorylated, active state, able to phosphorylate their target (Fujii et al. 2009). Later studies showed that SnRK2 activity can be further modulated by additional phosphorylation events (Vlad et al. 2010, Wang et al. 2013, Cai et al. 2014, Vilela et al. 2015). *In vivo* phosphorylation of SnRK2.2 and SnRK2.3 in positions 181 and 180, respectively (outside the T-loop) was initially reported in a phosphoproteomic study of ABA-treated Arabidopsis seedlings (Wang et al. 2013). Later findings let to the identification of BIN2 as a positive regulator of SnRK2s, potentiating their activity in response to ABA by phosphorylating Thr 180 (Cai et al. 2014). Inhibitory phosphorylation events in SnRK2s have also been reported. Casein kinase 2 (CK2) has been proposed as a

negative regulator of SnRK2s by phosphorylating several conserved Ser residues in a region defined as ABA box. These phosphorylation events, outside the T-loop, seem to strengthen the bond between SnRK2-PP2C and to induce SnRK2 proteasomal degradation (Vilela et al. 2015).

The family of PYR1/PYL/RCAR receptors comprises 14 members of soluble proteins in Arabidopsis (Ma et al. 2009, Park et al. 2009). *In vitro* and *in vivo* functional reconstitution assays in Arabidopsis protoplasts reveal that all of them, except PYL13, can function as ABA receptors and activate SnRK2 kinases (Fujii et al. 2009). At the subcellular level PYLs are mostly cytosolic and nuclear, but can be targeted to the plasma membrane in a calcium-dependent manner by interacting with CAR (C2-domain ABA-related) proteins, which positively regulate ABA sensitivity (Rodriguez et al. 2014).

Among the 76 PP2C phosphatases found in Arabidopsis, the nine clade A members act as core components of ABA signaling, serving as negative regulators (Schweighofer et al. 2004). At least six members of this clade have been shown to interact genetically with ABA-dependent SnRK2 kinases (Joshi-Saha et al. 2011). Moreover, immunoprecipitation of the ABI1 phosphatase fused with yellow fluorescent protein (ABI1-YFP) in Arabidopsis revealed the co-purification of nine PYR/PYL receptors as well as SnRK2.2 and SnRK2.3 kinases (Nishimura et al. 2010). An interaction between ABI1-YFP and SnRK2.6 had been previously reported employing a chemical cross-linker as stabilizer (Vlad et al. 2008), and was later confirmed *in vivo* also for two other PP2Cs, ABI2 and HAB1 (Vlad et al. 2009). In accordance with their role as negative regulators of ABA signaling, PP2C triple mutants (*hab/abi1/abi2* and *hab1/abi1/pp2ca*) show ABA hypersensitivity (Rubio et al. 2009).

3.3 ABA in short- and long-term stress responses

From the functional point of view, the stress responses mediated by ABA can be divided in short- and long-term responses. Short-term responses are related to stress acclimation and operate very rapidly (even within minutes of ABA accumulation), whereas long-term

responses are part of the developmental plasticity typical of plants in which ABA plays a dual role promoting or suppressing growth, at low and high concentrations, respectively (Humplik et al. 2017).

Comparative microarray analyses confirmed that, even at the transcriptomic level, long and short ABA treatment lead to remarkably different outputs (Yang et al. 2014). While short ABA exposure triggers differential expression of nearly 4500 genes, only around 2500 genes are differentially expressed by sustained ABA treatment (Yang et al. 2014). Moreover, a comparison between these two groups of genes shows only a partial overlap of about 1000 genes of which 210 are regulated in opposite ways by the two types of ABA treatments (Yang et al. 2014). Gene ontology analysis and quantitative real-time PCR (qPCR) of selected genes, indicate that a different crosstalk is established with other phytohormones (namely auxin, brassinosteroid, gibberellin, cytokinin, jasmonate and ethylene) in response to short and long ABA treatments (Yang et al. 2014).

3.3.1 Short-term responses to ABA

Since its discovery, ABA has been mostly associated to abiotic stresses such as drought and salinity in which it triggers stomata closure and the production of osmolytes to minimize water loss from cells (Zhu 2002).

Closure of stomata in response to ABA is a rapid response that can occur within seconds or minutes (Geiger et al. 2011) without requiring *de novo* transcription. Both in monocots and dicots, stomata are surrounded by a pair of bean-shaped guard cells, forming a cavity that allows gas exchange through the external layer of epidermis. Closure is achieved through an ancient hydraulic mechanism (Brodribb et al. 2011) in which the efflux of K^+ ions outside the plasma membrane induces water loss and thereby turgor pressure relaxation inside the guard cell and stomata closure (Munemasa et al. 2015). The cascade of events leading from ABA perception to rapid stomata closure involves classical components of the core ABA pathway and regulation of multiple downstream targets,

mainly ion channels, coordinated by the signal integration of different secondary messengers such as ROS and Ca^{2+} (Vahisalu et al. 2008, Kim et al. 2010, Lee et al. 2013, Munemasa et al. 2015, Murata et al. 2015). Among other members of subgroup III, Open Stomata 1 (OST1/SnRK2.6) plays an indispensable role in ABA perception in guard cells (Acharya et al. 2013). Active OST1 can rapidly phosphorylate and activate two classes of anion channels [slow (S)-type and rapid (R)-type] (Acharya et al. 2013, Imes et al. 2013) causing sustained plasma membrane depolarization which, in turn, activates voltage-dependent K^+ channel GUARD CELL OUTWARD RECTIFYING K^+ CHANNEL (GORK) (Hosy et al. 2003). K^+ is extruded through the open channel in accordance to its electrochemical gradient causing water loss and stomata closure. Core components of the ABA pathway are implicated in several layers of crosstalk between hormones and second messengers in the regulation of guard cell movements (Munemasa et al. 2015, Murata et al. 2015). For example, SnRK2 kinases are also required for the activation of S-type anion channels by Ca^{2+} dependent kinases (CDPKs) (Geiger et al. 2010, Brandt et al. 2015). Moreover, OST1 is able to trigger ROS signaling by activation of NAD(P)H oxidases RESPIRATORY BURST OXIDASE HOMOLOG (RBOH) (Acharya et al. 2013) and in doing so contributes to the regulation of stomata movement through the cross talk between ROS and Ca^{2+} second messengers (Murata et al. 2015, Sierla et al. 2016).

In addition to stomata closure, ABA triggers a rapid and vast transcriptional response that affects up to 25% of the Arabidopsis genes (Takahashi et al. 2004, Matsui et al. 2008, Zeller et al. 2009). Compared to other phytohormones (i.e. gibberellin, auxin, ethylene, cytokinin, brassinosteroid, and jasmonate), ABA has the largest impact on the transcriptome (Nemhauser et al. 2006).

Studies aimed at identifying ABA and stress-induced genes during vegetative growth revealed two waves of transcriptional responses (Finkelstein 2013). An early, transient response that peaks at 3 hours, and a late and sustained response that starts after 10 hours. “Early” genes encode proteins with regulatory functions in ABA signaling and effectors such as transcription factors, kinases, phosphatases, and early response to

dehydration (*ERD*) genes, many of which encode proteins of unknown function (Yamaguchi-Shinozaki et al. 2006, Fujita et al. 2011). “Late” genes codify mostly for proteins necessary to provide stress tolerance, such as ROS detoxifying components, enzymes of sugar or other osmo-compatible solute metabolism, ion and water-channel proteins, but also proteases and presumed chaperonins that help to maintain cellular integrity (Szabados et al. 2011, Kong et al. 2013).

Around 10% of the identified ABA-responsive genes are associated with transcription regulation (Nemhauser et al. 2006), including major classes of transcription factors such as bZIP, AP2/ERF, MYB, HB, zinc finger and WRKY (Kim 2014). Promoter analysis of ABA-responsive genes from different species led to the identification of *cis* acting elements known as ABA response elements (ABREs) (Menkens et al. 1995, Busk et al. 1998). Later “omics” approaches revealed that multiple ABRE elements are present in most of the ABA-induced genes (Yazaki et al. 2005, Zhang et al. 2005, Gomez-Porras et al. 2007). Several transcription factors of the basic (region) leucine zipper (bZIP) family were found to bind the ABRE element in yeast-one-hybrid experiments (Choi et al. 2000, Uno et al. 2000). bZIPs constitute a large family of transcription factors, comprising 78 members in Arabidopsis and classified in 13 groups (termed A-M) (Droge-Laser et al. 2018). Group A contains bZIPs that are activated by ABA and osmotic stress (Banerjee et al. 2017, Droge-Laser et al. 2018), and are generally named ABRE-binding factors (ABFs) or ABRE-binding proteins (AREBs). ABF1, ABF2/AREB1, ABF3, and ABF4/AREB2 were demonstrated to mediate stress and ABA responses *in vivo* (Fujita et al. 2005, Yoshida et al. 2010, Yoshida et al. 2015) and are considered direct targets of SnRK2 kinases and part of the core ABA signaling module (Uno et al. 2000, Fujii et al. 2009, Cutler et al. 2010, Yoshida et al. 2010, Fujita et al. 2013, Yoshida et al. 2015). *ABF1-4* are mostly expressed in vegetative tissues in response to abiotic stress conditions where ABA plays a critical function (Sakata Y. et al. 2014), namely high salinity, and osmotic (*ABF2-4*) and cold (*ABF1*) stress (Choi et al. 2000, Kang et al. 2002, Kim 2014). An increasing number of studies show however that ABA responses involve a complex network of TFs of various families that interact directly or indirectly with core components of ABA signaling (Kim 2014, Banerjee et al. 2017, Droge-

Laser et al. 2018). For example ABFs interacts with bZIPs of the C/S1 group (another large group involved in energy signaling (Droge-Laser et al. 2018)) during salt stress in roots (Hartmann et al. 2015) and interactions between the bZIP C/S1 group have also been reported with the ABI3 transcription factor (another core component of ABA signaling; see below) during seed maturation (Lara et al. 2003, Alonso et al. 2009).

3.3.2 Long-term responses to ABA – Developmental plasticity of root growth

Plant development occurs mostly post-embryonically, with new organs being generated from primary (SAM and RAM) and secondary meristems throughout the lifespan of the organism. The type of organs that are generated, as well as their number and characteristics are largely influenced by the environment, providing a developmental plasticity that maximizes fitness and survival. The process of branching dramatically shapes plant morphology both with regard to the aerial and underground parts, and in the case of the root is largely influenced by factors such as water availability, patches of nutrients or salts, physical obstacles, and the presence of micro-organisms or other plant roots (Linkohr et al. 2002, Zolla et al. 2010, Goh et al. 2013, Bao et al. 2014).

The root system consists of the primary root (PR), which is already defined during embryogenesis, and of secondary roots, which are formed post embryonically. These secondary roots can be divided into lateral roots (LRs), branching from the PR, and adventitious roots, which originate from other tissues (hypocotyl, stem, or even leaves). Root architecture is therefore largely determined by the activity of the primary and secondary root meristems (Sozzani et al. 2014). Although auxin is considered the primary hormone regulating meristem activity in the root (Overvoorde et al. 2010), ABA has also been shown to modulate root growth by controlling cell division and elongation (Spollen et al. 2000, De Smet et al. 2003, Zhang et al. 2010, Duan et al. 2013, Harris 2015). The effect of ABA on these processes, on the other hand, is largely dependent on the concentration applied, with high concentrations being inhibitory to growth whilst low

concentrations being stimulatory (Fujii et al. 2007, Dietrich et al. 2017, Humplik et al. 2017, Li et al. 2017). Another factor determining the ABA response is the tissue. In Arabidopsis primary and lateral roots display a different sensitivity to ABA and salt stress, with the latter being clearly more sensitive (De Smet et al. 2003, Duan et al. 2013). This different sensitivity contributes to shaping root architecture, allowing the primary root to grow and reach deeper soil districts at the expense of growth inhibition of lateral roots and the aerial parts (Sharp et al. 1988).

Using a candidate based approach several genes have been shown to negatively impact lateral root formation in Arabidopsis upon ABA and osmotic stress. Such genes control the ABA pathway at multiple level from PYR/PYLs (especially PYL8), to subclass III SnRK2s and ABA transcription factors, namely *ABI3*, *ABI4* and *ABI5* (Brady et al. 2003, De Smet et al. 2003, Shkolnik-Inbar et al. 2010, Zhao et al. 2014, Skubacz et al. 2016).

ABA is also implicated in shaping root development in response to resource availability. Hydrotropism, the directed root growth towards soil areas with higher water potential, is a process depending on multiple components of the core ABA module such as SnRK2.2, PP2Cs and the PYL8 receptor (Antoni et al. 2013, Dietrich et al. 2017, Belda-Palazon et al. 2018). Interestingly, hydrotropic signaling happens by repressing gravitropic growth and the signals regulating the two types of tropism originate from different root zones, transition and elongation zone for hydrotropism (Dietrich et al. 2017) and columella cells at the root tip for gravitropism (Blancaflor et al. 1998).

3.4 Role of ABA in development

Plants deficient in ABA synthesis display phenotypic abnormalities even in well-watered growth conditions or high relative humidity, and these defects are rescued by low concentrations of exogenous ABA, revealing that ABA is required for normal plant development (Barrero et al. 2005). Even when grown in optimal conditions, leaves and rosettes of *aba1* mutants are smaller than wild-type, leaves curl downwards can be

occasionally lobed (Barrero et al. 2005). ABA impinges on development by interacting at multiple levels with other hormonal pathways such as brassinosteroid, gibberellin, cytokinin, and auxin (Zhang et al. 2009, Huang et al. 2018, Shu et al. 2018), therefore affecting plant development from seed germination (see below) to flowering and senescence (Zhao et al. 2017, Shu et al. 2018). The role of ABA in controlling floral transition remains elusive since both effects of flowering-promoting and flowering-inhibiting have been reported for the same molecular components (Shu et al. 2018). To solve this dichotomy a functional explanation has been proposed which takes into account the origin of the ABA produced. When ABA signaling is activated by overexpression of key ABA effector genes it plays an inhibitory role, whereas when ABA accumulation is a result of drought stress, ABA has a flowering promoting role (Riboni et al. 2013, Wang et al. 2013, Shu et al. 2016, Shu et al. 2018). These observation suggest that the severity of drought stress can determine which role on flowering time will be executed by ABA (Shu et al. 2018).

The best characterized functions of ABA in development relate to the control of embryo maturation, the establishment of seed dormancy and germination.

3.4.1 Embryo desiccation and seed maturation

In angiosperms, the last step of embryogenesis is concluded when the embryo reaches the heart phase in which the primary meristems are defined. At this stage, developing embryos enter the maturation phase by shifting from growth based on cell division to cell expansion and the accumulation of reserve compounds (Capron et al. 2009, Finkelstein 2013). During seed maturation, there are two peaks of ABA accumulation. In an initial phase maternally` synthetized ABA is required to stall the cell cycle of embryo cells in G1/S transition phase (Marisa Levi et al. 1992), potentially through the induction of *ICK1*, encoding a cyclin-dependent kinase (CDK) inhibitor (Wang et al. 1998). This first exposure to ABA avoids premature embryo germination and initiates the accumulation of reserves

that will sustain the initial growth phases after germination. Indeed, the *aba1 abi3* double mutant has reduced accumulation of storage proteins and oils (Meurs et al. 1992, Bruijn et al. 1997), while application of exogenous ABA promotes the accumulation of soluble sugars and lipids in seeds of *Ricinus communis* (Chandrasekaran et al. 2014). Many genes codifying storage proteins harbor in their promoter region *cis*-acting elements that respond to ABA, and are regulated through the coordinated action of different transcriptional regulators (Nakashima et al. 2013).

One such regulator is ABA INSENSITIVE 3 (ABI3), an AP2/B3-like family transcription factor (Monke et al. 2012). Several storage protein genes such as *At2S3/SEED STORAGE ALBUMIN 3* (*SESA3*), *CRUCIFERIN C* (*CRC*) and *LEAFY COTYLEDON 1* (*LEC1*)-mediated seed storage protein are regulated by ABI3 in an ABA-dependent manner during seed maturation (Santos-Mendoza et al. 2008). Genome-wide chromatin immunoprecipitation (ChIP-chip) experiments led to the identification of 98 targets of ABI3, many of which involved in storage protein and lipid accumulation (Monke et al. 2012). ABA INSENSITIVE 4 (ABI4) is another important positive regulator of ABA signaling, essential for lipid biosynthesis during embryo maturation. Lipids accumulate in the form of triacylglycerols (TAGs), esters of glycerol and fatty acids. ABI4 was shown to be required for the induction of *DGAT1* (acyl-coenzyme A: diacylglycerol acyltransferase1), encoding a rate-limiting enzyme for TAG synthesis (Yang et al. 2011). DGAT1 activation is also dependent on ABI5, which functions synergistically with ABI4 in *DGAT1* transcription in response to stress (Kong et al. 2013). Interestingly, ABI3 and ABI4 ectopic expression is sufficient to induce the production of seed storage proteins and lipids in vegetative tissues (Soderman et al. 2000, Kagaya et al. 2005).

The second peak of ABA accumulation is produced directly by the embryo during a later phase and is required for conferring desiccation tolerance and for inducing dormancy (Finkelstein 2013). Seed dormancy has been defined as a quiescent state of a viable seed that is incapable of germinating under favorable conditions (Bewley 1997). This dormant state ensures seed viability under adverse environmental conditions. Seed viability during

desiccation (which can vary from 1-10% of water content (Manfre et al. 2009)) is achieved by the progressive accumulation of compounds that protect the integrity of cells and organelles and that prevent unspecific protein aggregation during the late phases of seed maturation (Hoekstra et al. 2001). Such protective compounds include non-reducing sugars (Black et al. 1999, Buitink et al. 2000) and proteins like small heat shock proteins (HSPs) and late embryogenesis abundant proteins (LEA).

In accordance with the role of ABA in establishing dormancy, mutants of ABA biosynthesis and signaling produce non-dormant seeds or seeds with reduced dormancy (Koornneef et al. 1982, Finkelstein 1994, Nambara et al. 1995, Leon-Kloosterziel et al. 1996, Raz et al. 2001, Lefebvre et al. 2006, Gutierrez et al. 2007). An extreme example of this are triple *snrk2.2 snrk2.3 snrk2.6* mutants which are completely insensitive to ABA and their non-dormant seeds germinate precociously inside the siliques when humidity levels are adequately high (Fujita et al. 2009, Nakashima et al. 2009). A similar viviparous phenotype was reported in maize for several *viviparous* (*vp*) mutants which are impaired in ABA synthesis, or perception as the case of *vp1*, the orthologue monocots of *abi3* (McCarty et al. 1989, McCarty 1995). In Arabidopsis, vivipary has been found in double mutants combining *fus3* with ABA deficiency (e.g. *aba1* or *aba2*) or impaired ABA signaling (e.g. *abi1*, *abi3*, *abi4* or *abi5*) (Nambara et al. 2000, Raz et al. 2001, Finkelstein 2013), suggesting that the appearance of this phenotype requires mutations in two distinct pathways involving both maternal and embryonic tissues (Raz et al. 2001).

3.4.2 Germination

After a certain period of storage, dormancy declines and the seeds become receptive to environmental stimuli (Finkelstein 2010). Low temperatures and exposure to light are the major factors that release seed dormancy and enable completion of germination (Holdsworth et al. 2008). In Arabidopsis seeds, dormancy can be released by exposing seeds to cold temperature while imbibing (stratification), a treatment that mimics the

winter period and allows germination when seeds are returned to optimal temperatures. ABA levels decline rapidly within 3-18 h upon imbibition (Linkies et al. 2009, Preston et al. 2009) which is an indispensable event for seeds germination (Weitbrecht et al. 2011). ABA reduction is mainly caused by the activity of *CYP707A2* (*CYTOCHROME P450*) encoding ABA 8'-hydroxylase responsible for ABA catabolism in seeds (Kushiro et al. 2004). In the presence of ABA germination is prevented by the action of ABA-dependent transcription factors, mainly ABI3, ABI4 and ABI5 (Finkelstein 2013, Yan et al. 2017). A tight interplay between ABA and gibberellin ensures that germination takes place in suitable conditions (Jacobsen et al. 2002, Holdsworth et al. 2008, Daszkowska-Golec 2011). High levels of ABA associated with low levels of gibberellin prevent germination under unfavorable conditions, while low levels of ABA and high levels of gibberellin promote this process (Liu et al. 2014, Vishal et al. 2018).

3.4.3 Post germination growth arrest

After germination, the young seedlings can still undergo growth arrest mediated by ABA. Once germinated, the embryo is still sensitive to ABA during a limited time window of ~48 h (Lopez-Molina et al. 2001, Lopez-Molina et al. 2002). Arabidopsis seedlings eventually germinate even in presence of ABA, but their development arrests prior to cotyledon expansion and greening (Lopez-Molina et al. 2001, Kinoshita et al. 2010). This post-germination developmental arrest represents a protective mechanism that prevents the transition from germination to the more stress-sensitive vegetative growth stage under adverse environmental conditions. In response to water shortage, or osmotic stress, ABA levels increase, arresting the vegetative growth program before cotyledons green. As a consequence, the seedling remains in a quiescent stage, resembling the reinitiation of an embryogenic program that prevents the transition to the stress-vulnerable seedling state. In fact, growth arrested embryos start *de novo* expression of *LEA* genes (for example *AtEm1* and *AtEm6*), which confer osmotolerance as long as ABA is present (Finkelstein 1993, Lopez-Molina et al. 2001, Lopez-Molina et al. 2002). During the

past years, several studies started to shed light on the genetic and molecular processes allowing plant developmental plasticity this time window (Lopez-Molina et al. 2001, Lopez-Molina et al. 2002, Miura et al. 2009, Kinoshita et al. 2010, Hu et al. 2014, Albertos et al. 2015, Wu et al. 2019). Screens for insensitivity to ABA (3 µM) lead to the identification of two recessive DNA *loci* mediating post-germinative development arrest, *GIA1* (Growth Insensitive to ABA - a novel allele of *ABI5*) and *GIA3* (Lopez-Molina et al. 2000, Lopez-Molina et al. 2001, Kinoshita et al. 2010). The two mutations appear to affect two distinct gene functions, as demonstrated by genetic crosses between *abi5* and *gia3* mutants (Kinoshita et al. 2010). Phenotypical analysis of the single and double *abi5*/*gia3* reveal that certain aspects of ABA-dependent developmental arrest are commonly regulated by the two *loci* and others appear specific to a single *locus*. Indeed, while both single mutants equally accumulate more chlorophyll in presence of ABA compared to wild type (and the double mutant show additive ABA insensitivity compared to single mutants), other features such as the inhibition cotyledons expansion can be ascribed solely to *gia3* as confirmed by the observation that the double *abi5*/*gia3* mutant is as insensitive as single *gia3* (the single *abi5* mutant is similar to wild type concerning this type of ABA response) (Kinoshita et al. 2010). Functional and phenotypical analysis indicate that ABI5 acts downstream of ABI3 since the *abi5* mutation is able to rescue the ABA-insensitivity of the *abi3* mutant, while the ABI3 overexpression (known to confer ABA hypersensitivity) cannot revert the ABA-insensitivity of the *abi5* mutant line (Lopez-Molina et al. 2002).

Noteworthy, the components that mediate the ABA-dependent post-germination growth arrest are also involved in ABA responses in other developmental stages, as reported for endosperm rupture during germination and other stages of vegetative growth or phase transitions (Finkelstein 1994, Kinoshita et al. 2010, Kong et al. 2013, Wang et al. 2013, Skubacz et al. 2016) reinforcing the idea that plant developmental plasticity is accompanied by functional plasticity.

Research objective and thesis outline

The main objective of the work presented in this thesis is focused on the regulation of SnRK1 in the model plant *Arabidopsis thaliana*. This evolutionary conserved energy sensor plays a central role in the metabolic and transcriptional response to energy starvation at the cellular and at the whole plant level. Considering that numerous stress forms negatively impact on energy metabolism resulting in energy imbalance, SnRK1 is involved in virtually all kind of stress responses. In addition to its role in regulating energy stress, SnRK1 plays pivotal roles during plant developmental transitions spanning from germination to flowering, fruit ripening and senescence. SnRK1 is therefore a research target of several fundamental research lines, but also of direct applications aimed at crop yield improvement.

Despite the numerous basic and applied studies, little is known about the molecular mechanisms of SnRK1 regulation. The emerging picture indicates that, although the high levels of conservation with its yeast and mammals counterparts in the downstream effects, plant SnRK1 presents unique regulatory features that need to be further investigated.

Experimental Chapters:

Chapter II - ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in Arabidopsis.

In this chapter, is reported the work in which two PP2C proteins phosphatases were identified as physiological negative regulators of SnRK1 signaling. ABI1 and PP2CA are two representative members of clade A PP2Cs that are established negative regulators of SnRK2 kinases, the positive effectors of ABA signaling. A combination of functional,

genetic and biochemical approach was employed to demonstrate that these two phosphatases are acting as *in planta* negative regulators of SnRK1. As a consequence of this, the SnRK1 transcriptional response results to be activated by ABA in a PP2C-dependent manner. Moreover, clade A PP2C phosphatases are necessary to extinguish SnRK1 signaling in response to glucose.

Chapter III - A dual function of SnRK2 kinases in plant growth regulation.

In this chapter is presented the work in which the role of SnRK1 in the ABA pathway has been investigated in molecular details. Thanks to the generation of stable SnRK1 *knock-down* line it was confirmed that the ABA-dependent growth arrest is dependent on a fully operational SnRK1 signaling pathway. Further analysis indicate that such ABA-insensitive phenotype is caused by defects in the inhibition of the growth-promoting complex, TOR. A Recent work shown that the ABA-dependent TOR inactivation is strictly dependent on the action of the SnRK1-related SnRK2 kinases. Here, genetic and biochemical evidences support the idea that SnRK1, and not SnRK2s, is actually interacting with the TOR complex leading to its inhibition, most likely, by phosphorylating RAPTOR in response to ABA. Moreover, in control conditions, a SnRK2s mutant line displays growth defect phenotype in roots which is dependent on SnRK1 as indicated by genetic crosses. Collectively, the results obtain in ABA and control conditions lead us to propose a dual role of SnRK2 kinases in the regulation of SnRK1 and its downstream targets. In optimal growth conditions, SnRK2s act as negative regulators of SnRK1 participating to the formation of repressed SnRK1 complexes. In presence of ABA, they convey the ABA signaling to SnRK1-SnRK2 complexes releasing SnRK1 from repression. ABA-activated SnRK1, in turn, is mediating TOR inactivation in response to the hormone.

Chapter IV – Conclusions.

In this Chapter, the main findings of the Experimental Chapters are reviewed in the light of the current knowledge on SnRK1 regulation, and their biological relevance is discussed. Future research directions are proposed to tackle new questions arisen from Chapter II and Chapter III.

Chapter II - *ABI1 and PP2CA Phosphatases Are Negative Regulators of Snf1-Related Protein Kinase1 Signaling in Arabidopsis*

This chapter was published in:

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Main text

Plant survival under environmental stress requires the integration of multiple signaling pathways into a coordinated response, but the molecular mechanisms underlying this integration are poorly understood. Stress-derived energy deprivation activates the Snf1-related protein kinases1 (SnRK1s), triggering a vast transcriptional and metabolic reprogramming that restores homeostasis and promotes tolerance to adverse conditions. Here, we show that two clade A type 2C protein phosphatases (PP2Cs), established repressors of the abscisic acid (ABA) hormonal pathway, interact with the SnRK1 catalytic subunit causing its dephosphorylation and inactivation. Accordingly, SnRK1 repression is abrogated in double and quadruple *pp2c* knockout mutants, provoking, similarly to SnRK1 overexpression, sugar hypersensitivity during early seedling development. Reporter gene assays and SnRK1 target gene expression analyses further demonstrate that PP2C inhibition by ABA results in SnRK1 activation, promoting SnRK1 signaling during stress and once the energy deficit subsides. Consistent with this, SnRK1 and ABA induce largely overlapping transcriptional responses. Hence, the PP2C hub allows the coordinated activation of ABA and energy signaling, strengthening the stress response through the cooperation of two key and complementary pathways.

Changes in water and nutrient availability, soil salinity, and extreme temperatures, among others, generate signals in plants that need to be finely integrated with metabolic activity and development for optimal growth and survival (Smith and Stitt, 2007). One such signal is energy deficiency derived from impaired carbon assimilation and/or respiration in situations of stress, which triggers the activation of the SnRK1 protein kinases to restore homeostasis and elaborate adequate longer term responses through a vast metabolic and transcriptional reprogramming (Radchuk et al., 2006; Schwachtje et al., 2006; Baena-

González et al., 2007; Baena-González and Sheen, 2008; Lee et al., 2009). The *Arabidopsis thaliana* genome encodes 38 SnRKs, of which three, SnRK1.1 (KIN10/AKIN10), SnRK1.2 (KIN11/AKIN11), and SnRK1.3 (KIN12/AKIN12), represent the orthologs of the budding yeast (*Saccharomyces cerevisiae*) sucrose-nonfermenting1 (Snf1) and mammalian AMP–activated protein kinase (AMPK) metabolic sensors (Halford et al., 2003; Polge and Thomas, 2007; Hardie, 2011). An increasing body of evidence suggests that SnRK1s act as convergence points for various metabolic, hormonal and stress signals during growth and development, linking it to key hormonal pathways and in particular to abscisic acid (ABA; Németh et al., 1998; Bhalerao et al., 1999; Bradford et al., 2003; Radchuk et al., 2006; Baena-González et al., 2007; Lu et al., 2007; Rosnoblet et al., 2007; Ananieva et al., 2008; Baena-González and Sheen, 2008; Lee et al., 2008; Jossier et al., 2009; Radchuk et al., 2010; Coello et al., 2012; Tsai and Gazzarrini, 2012). SnRK1 is a heterotrimeric complex composed of an α-catalytic subunit (SnRK1.1/1.2/1.3 in Arabidopsis) and two regulatory subunits, β and γ (Polge and Thomas, 2007). Similarly to its mammalian and yeast counterparts, SnRK1 activity requires phosphorylation of a highly conserved T-loop residue (T175 in SnRK1.1) (Estruch et al., 1992; Hawley et al., 1996; Stein et al., 2000; McCartney and Schmidt, 2001; Baena-González et al., 2007; Shen et al., 2009; Crozet et al., 2010). Under normal energy conditions in mammalian cells, MgATP is bound to the γ subunit of the AMPK complex resulting, through the joint action of the constitutively active upstream liver kinase B1 and the still unknown upstream phosphatase, in a basal T-loop phosphorylation:dephosphorylation cycle with no net AMPK activation (Hardie, 2011). Under energy deficiency conditions, the replacement of MgATP by AMP/ADP triggers a conformational change that promotes AMPK phosphorylation and, most importantly, protects AMPK from dephosphorylation by rendering it a poor substrate for phosphatases (Oakhill et al., 2011; Xiao et al., 2011). Despite the rate of dephosphorylation being a primary determinant of AMPK activity, the identity of the AMPK phosphatase(s) remains unclear and may differ between tissues and conditions of cell stimulation (Steinberg and Kemp, 2009; Carling et al., 2012). In budding yeast, Reg1, a regulatory subunit of the type 1 protein phosphatase Glc7, interacts with Snf1 and is required to maintain Snf1 in an inactive state during growth on Glc (Sanz et al., 2000;

Hong et al., 2005). The metabolic signal underlying Snf1 regulation remained enigmatic for a long time, but recent work demonstrated that Snf1 is also regulated by ADP at the substrate level, preventing its dephosphorylation by phosphatases (Mayer et al., 2011). In plants, SnAK1/2 (also called Geminivirus Rep interacting kinase 2/1) have been identified as upstream SnRK1 kinases (Shen et al., 2009; Crozet et al., 2010), but the phosphatases responsible for resetting SnRK1 signaling are unknown.

In Arabidopsis, at least seven of the nine type 2C protein phosphatases (PP2Cs) from clade A (Schweighofer et al., 2004) act as negative regulators of the ABA pathway (Gosti et al., 1999; Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004, 2006; Kuhn et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007; Rubio et al., 2009; Antoni et al., 2012) through their interaction with SnRK2s, more divergent members of the SnRK family and specific to plants (Halford et al., 2003; Cutler et al., 2010). Arabidopsis contains 10 SnRK2s, of which three, SnRK2.2/2.3/2.6, are specifically activated by ABA and play a central role in the ABA pathway (Gómez-Cadenas et al., 1999; Li et al., 2000; Mustilli et al., 2002; Boudsocq et al., 2004, 2006; Yoshida et al., 2006; Fujii et al., 2007, 2009). Clade A PP2Cs regulate SnRK2.2/2.3/2.6 through physical obstruction and direct dephosphorylation of a conserved Ser residue in the T-loop (S175 in SnRK2.6) (Umezawa et al., 2009; Vlad et al., 2009; Soon et al., 2012). In the presence of ABA, the Pyrabactin Resistance1/Pyrabactin Resistance1-Like (PYL)/Regulatory Components of ABA Receptors family of ABA receptors (hereafter PYL) inhibit PP2Cs, resulting in SnRK2 activation and downstream gene expression (Ma et al., 2009; Park et al., 2009; Soon et al., 2012).

Considering that clade A PP2Cs, through interaction with a wide array of targets, act as a regulatory hub for different abiotic stress responses (Sheen, 1996; Chérel et al., 2002; Guo et al., 2002; Himmelbach et al., 2002; Ohta et al., 2003; Miao et al., 2006; Yang et al., 2006; Umezawa et al., 2009; Vlad et al., 2009; Geiger et al., 2010) and taking into account the role of SnRK1 as a convergence point for multiple types of stress (Baena-González et al., 2007), we postulated that clade A PP2Cs might function as SnRK1 phosphatases. An additional hint came from data mining on a high-throughput proteomics screen for yellow

fluorescent protein (YFP)-ABI1-interacting proteins, which inadvertently identified SnRK1s as putative ABI1-interacting proteins (Nishimura et al., 2010) (see below).

Here, we provide molecular, genetic, and physiological evidence for the role of two clade A PP2Cs, ABI1 and PP2CA, as negative regulators of SnRK1 signaling in Arabidopsis through their direct interaction with the SnRK1 α-catalytic subunit, its dephosphorylation, and subsequent inactivation, hence contributing to resetting SnRK1 signaling upon the remittance of stress. In contrast, PP2C inhibition allows ABA to promote SnRK1 activity, potentiating the stress response through the interplay of two complementary pathways and providing an explanation for the extensive genetic interactions reported between ABA and sugar signaling (Rolland et al., 2006).

RESULTS:

ABI1 and PP2CA Interact with the SnRK1 Catalytic Subunit

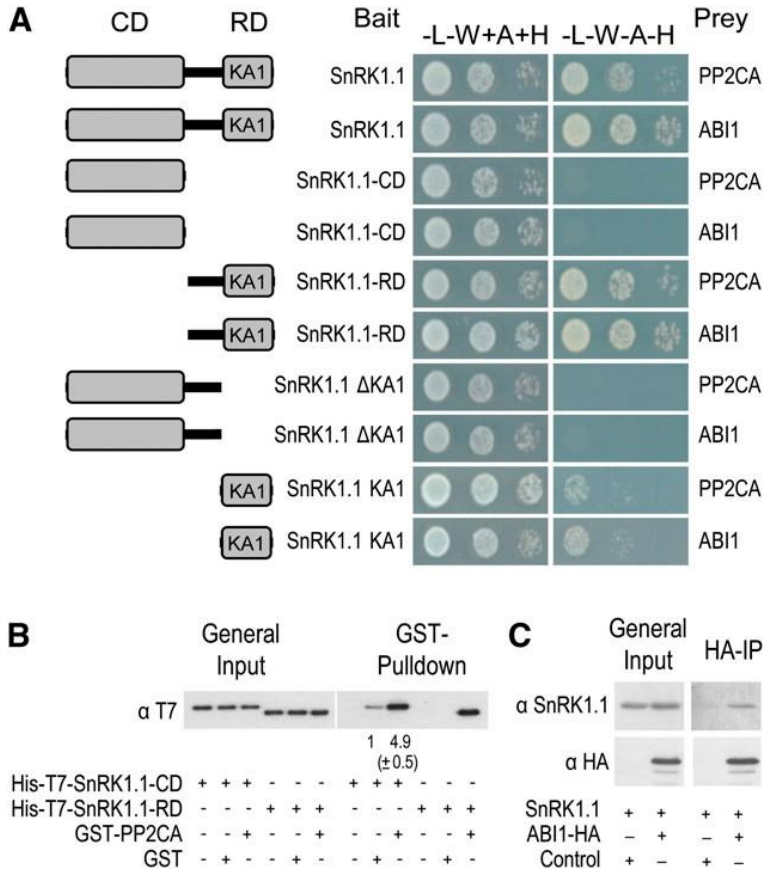
A high-throughput screen using green fluorescent protein (GFP) – affinity purification and mass-spectrometric analyses was performed by Nishimura and colleagues to identify proteins interacting with YFP-ABI1 (Nishimura et al., 2010). Data mining of their results revealed the presence of peptides corresponding to both SnRK1s in several of their replicate experiments with YFP-ABI1 (SnRK1.1 in experiments 1, 3, and 8 and SnRK1.2 in experiments 1 and 3), whereas neither of the two SnRK1s was identified in any of the YFP control experiments.

As a first step to validate these data and investigate the possible regulation of SnRK1 by clade A PP2Cs, we tested in yeast two-hybrid (Y2H) assays the interaction between the SnRK1 catalytic subunit and ABI1 or PP2CA, representative members of the two clade A branches in the PP2C family (Schweighofer et al., 2004). SnRK1.1 interacted with ABI1 and PP2CA in yeast cells, and deletion of its regulatory domain (RD) abolished this interaction (Figure 1A; Supplemental Figure 1A). The N terminus harbors the kinase catalytic domain

(CD), whereas the C terminus harbors the RD that binds the β and γ subunits (Polge and Thomas, 2007). The SnRK1 RD contains a subdomain of unknown function, the kinase-associated1 (KA1) domain, that was reported in the SnRK3.11/Salt Overly Sensitive2 (SOS2) protein kinase to closely superimpose on the protein phosphatase interaction domain (Sánchez-Barrena et al., 2007), a docking site for the clade A PP2C ABI2 (Ohta et al., 2003). Modeling SnRK1.1 with the structures resolved for the KA1 domain in SnRK3.11 (Sánchez-Barrena et al., 2007), the AMPK-related microtubule-affinity–regulating kinase3 (Tochio et al., 2006), and for AMPKa (Xiao et al., 2011), revealed that in SnRK1.1, this subdomain spans residues 390 to 512 (Supplemental Figure 2). As shown, the KA1 domain was both required and sufficient for the interaction with the phosphatase (Figure 1A). Nevertheless, colony growth when using the KA1 domain alone was weaker than with SnRK1.1-RD or the full-length protein, suggesting that other regions may play a role in the PP2C interaction.

To further validate the Y2H data, we performed an in vitro pull-down assay (Figure 1B). Purified recombinant His-SnRK1.1-CD or His-SnRK1.1-RD was incubated with glutathione S-transferase (GST)—PP2CA, GST, or the beads and the interacting proteins were pulled down using a glutathione–agarose matrix. SnRK1.1-RD was recovered only when using GST-PP2CA as bait. In the case of SnRK1.1-CD, a fivefold enrichment was observed when using GST-PP2CA compared with GST alone, suggesting that even though not detected in the Y2H assay, PP2Cs interact also to some degree with the SnRK1.1-CD. No SnRK1-RD or SnRK1.1-CD was recovered from the beads alone. To determine whether a SnRK1.1-PP2C interaction occurs also in planta, SnRK1.1 was transiently coexpressed in Arabidopsis protoplasts with control DNA or with a plasmid expressing ABI1-hemagglutinin (HA). Immunoprecipitation with an anti-HA antibody revealed a specific interaction between SnRK1.1 and ABI1-HA (Figure 1C), demonstrating that ABI1 also interacts with SnRK1.1 in vivo.

Figure 1. ABI1 and PP2CA Interact with SnRK1.1 in vitro and in vivo.



(A) SnRK1.1 interacts with ABI1 and PP2CA in Y2H assays. Protein interaction was determined by growth assay in medium lacking Leu, Trp, adenine and His (2L2W2A2H) compared with control medium lacking Leu and Trp but supplemented with adenine and His (2L2W+A+H). **(B)** In vitro interaction between GST-PP2CA and His-T7-SnRK1.1 detected by GST pull down and T7 immunodetection of SnRK1.1 preys. Numbers below immunoblot denote band intensities compared with GST-alone control (=1); values represent means 6SD (*n* = 3). **(C)** HA immunoprecipitation pulls down SnRK1.1 from protoplasts co-expressing SnRK1.1 (untagged) with ABI1-HA, but not with control DNA.

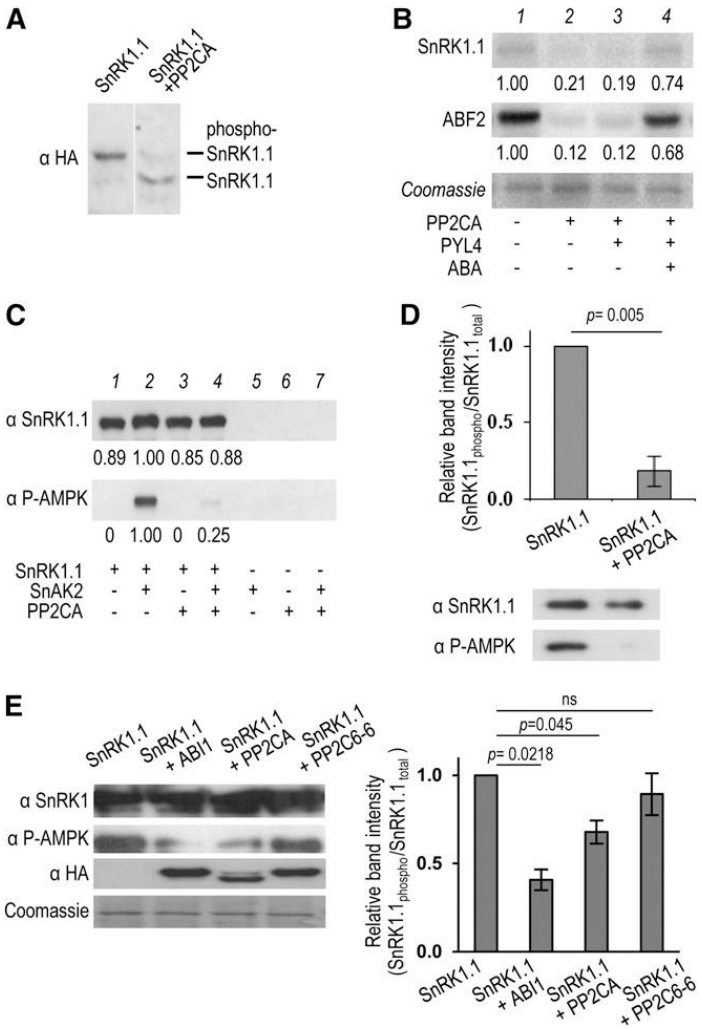
ABI1 and PP2CA Dephosphorylate and Inactivate SnRK1.1

To evaluate whether the detected PP2C-SnRK1.1 interaction results in SnRK1.1 dephosphorylation and inactivation, we immunoprecipitated SnRK1.1 from plants overexpressing an HA-tagged version (35S:SnRK1.1-HA) (Baena-González et al., 2007) and treated with recombinant His-PP2CA. PP2CA treatment caused a clear dephosphorylation of SnRK1.1, as assessed by a faster mobility in a Phos-Tag SDS-PAGE that selectively retards phosphorylated proteins (Kinoshita et al., 2009) (Figure 2A). To investigate the effect of this dephosphorylation on SnRK1 activity, we performed in vitro kinase assays. In agreement with previous reports, active SnRK1.1 could efficiently autophosphorylate and phosphorylate the Abscisic acid responsive elements-Binding Factor2 (ABF2) transcription factor in vitro (Bhalerao et al., 1999; Zhang et al., 2008; Shen et al., 2009) (Figure 2B, lane 1). No ABF2 phosphorylation could be observed in control HA pull downs from wild-type (WT) plants, confirming that the measured activity corresponds to SnRK1-HA (see Supplemental Figure 3A online). Addition of PP2CA to the reaction caused a substantial decrease in the phosphorylation of both SnRK1.1 and ABF2 (Figure 2B, lane 2). The PYL receptors inhibit clade A PP2Cs in the presence of ABA, resulting in SnRK2 activation (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009). Adding the PYL4 receptor in the absence of ABA did not change the ability of PP2CA to inactivate SnRK1 (Figure 2B, lane 3), whereas in the presence of ABA, PYL4 fully blocked SnRK1.1 inactivation by PP2CA (Figure 2B, lane 4). To rule out the possibility that decreased ABF2 phosphorylation in the presence of PP2CA results from direct ABF2 dephosphorylation by PP2CA rather than from lower SnRK1 activity, SnRK1.1 was preincubated with PP2CA and PYL4 in the absence (PP2CA active) or presence (PP2CA inactive) of ABA (Supplemental Figure 3B, lanes 2 and 3,). Following this incubation, ABA was added to block further PP2CA action before the addition of ABF2. Preincubation of SnRK1 with PP2CA in the absence of ABA resulted in undetectable SnRK1 activity and ABF2 phosphorylation, suggesting that the effect of PP2CA on ABF2 phosphorylation was at least partly due to a reduction in SnRK1 activity rather than to a direct dephosphorylation of ABF2 by the phosphatase.

SnRK1 requires phosphorylation of the T-loop T175 residue for activity (Baena-González et al., 2007; Shen et al., 2009; Crozet et al., 2010). To test whether T175 could be a substrate for ABI1 and PP2CA, we first performed in vitro dephosphorylation experiments. Recombinant SnRK1.1 is not phosphorylated and hence is barely active but it can be strongly activated by the upstream kinases SnAK1/2 through the specific phosphorylation of T175 (Shen et al., 2009; Crozet et al., 2010). GST-PP2CA treatment of recombinant GST-SnRK1.1, prephosphorylated with GST-SnAK2, resulted in significant T175 dephosphorylation, as detected with an anti-phospho-AMPKa(T172) (T172) antibody (Sugden et al., 1999; Baena-González et al., 2007) (Figure 2C) that specifically recognizes SnRK1.1 and SnRK1.2 phosphorylated in the T-loop (T175 for SnRK1.1; see Supplemental Figure 4 online). A similar effect was observed when SnRK1.1 was immunoprecipitated from 35S:SnRK1.1-HA plants and treated with GST-PP2CA (Figure 2D), altogether showing that T175 is efficiently dephosphorylated by PP2Cs in vitro.

To determine whether T175 is a PP2C substrate in vivo, we used Arabidopsis mesophyll protoplasts to transiently express SnRK1.1-GFP alone or in combination with various PP2Cs. As shown in Figure 2E, coexpression of SnRK1.1-GFP with either ABI1 or PP2CA (from clade A) resulted in a significant reduction in T175 phosphorylation levels, while coexpression with the unrelated PP2C6-6 from clade E (Schweighofer et al., 2004) did not have an impact on T175 phosphorylation. These results suggest that T175 is a substrate for ABI1 and PP2CA also in vivo.

Figure 2. ABI1 and PP2CA Inhibit SnRK1.1 by Dephosphorylation.



Immunoprecipitated SnRK1.1-HA is dephosphorylated (A) and inactivated (B) in vitro by PP2CA. **(A)** HA immunoblot following Phos-Tag-SDS-PAGE (Kinoshita et al., 2009). **(B)** Autoradiograms showing that SnRK1.1 activity on itself and ABF2 (lane 1) is lost following His-PP2CA-treatment (lane 2) but rescued by PYL4 and ABA (lane 4). GST-PP2CA dephosphorylates T175 in recombinant SnRK1.1, phosphorylated or not with SnAK2 **(C)**, and in immunoprecipitated SnRK1.1 (n = 3) **(D)** in vitro. Numbers below autoradiograms and immunoblots denote band intensities relative to SnRK1.1 control (=1). At least three independent experiments were performed in (A) to (C) with similar results. **(E)** Coexpression in protoplasts of SnRK1.1 with clade A PP2Cs ABI1 and PP2CA, but not with clade E PP2C6-6, results in SnRK1.1 (T175) dephosphorylation. PP2Cs and SnRK1.1 bear HA and GFP tags, respectively. SnRK1.1 (T175) phosphorylation was

detected by immunodetection with anti-phospho-AMPKα (T172) antibodies (n = 6). Error bars = SE; P values, two-tailed paired Student's t test (D) and one-way ANOVA with Tukey test (E) on the non-normalized ratio of SnRK1.1(T175) phosphorylation relative to total SnRK1.1.

ABI1 and PP2CA Repress SnRK1 Signaling

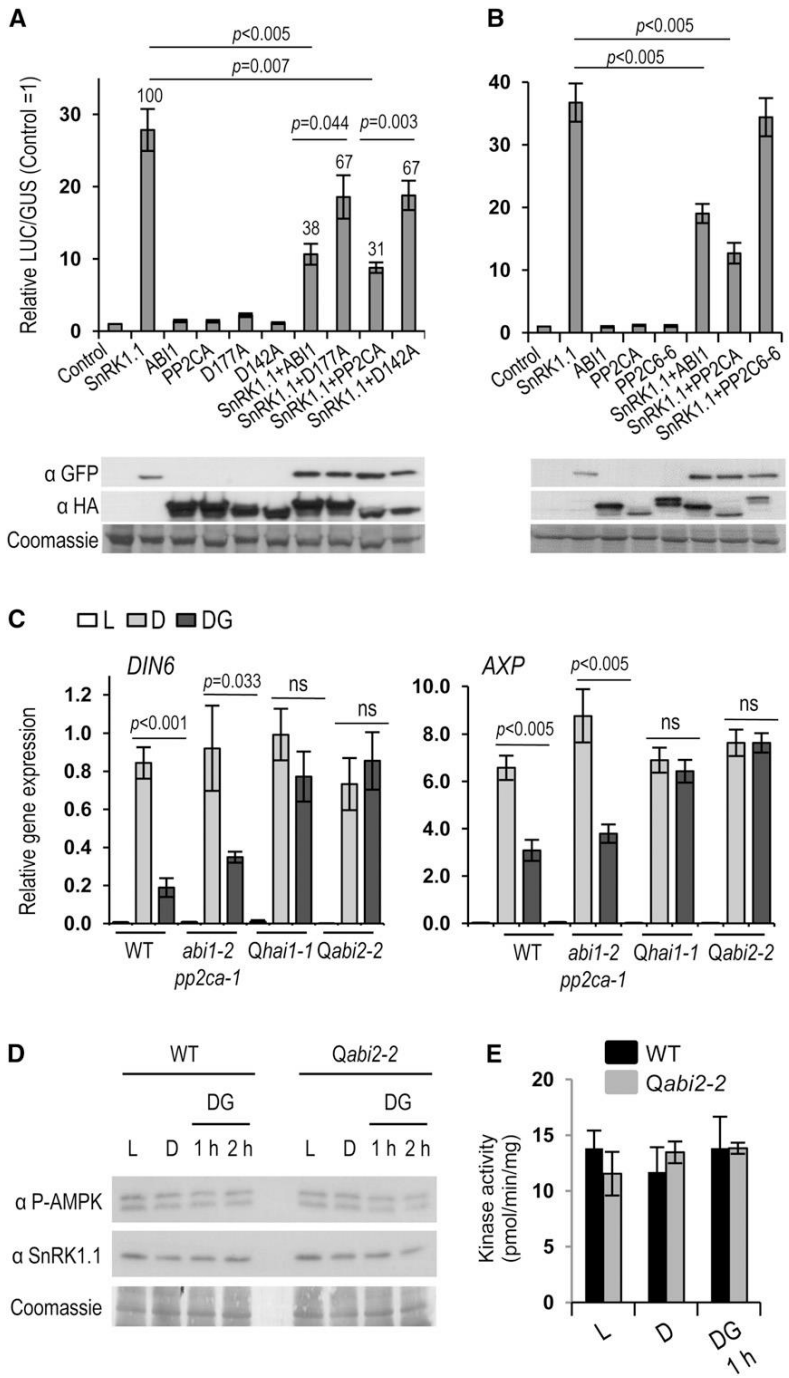
To further explore the functional implications of SnRK1 regulation by PP2Cs, we employed a transient cell-based assay that uses luciferase (LUC) induction from the DIN6:LUC reporter as a read-out of SnRK1 activity (Baena-González et al., 2007). In transfected mesophyll protoplasts, SnRK1.1 overexpression is sufficient to induce strong LUC activity under control conditions (Figure 3A) (Baena-González et al., 2007). Coexpression with the ABI1 or PP2CA phosphatases reduced SnRK1.1-mediated DIN6:LUC induction by 60% without affecting SnRK1.1 levels (Figure 3A). Importantly, the ability of these phosphatases to repress reporter gene induction by SnRK1.1 was strongly diminished in the corresponding catalytically inactive variants (ABI1_D177A and PP2CA_D142A; Figure 3A), suggesting that repression of SnRK1 signaling by ABI1 and PP2CA occurs to a large extent through dephosphorylation. As a negative control, co-expression with the unrelated PP2C6-6 from clade E (Schweighofer et al., 2004) had no significant effect on the ability of SnRK1.1 to induce the reporter (Figure 3B), altogether supporting the specific repressive role of ABI1 and PP2CA on the SnRK1 pathway.

To investigate the influence of ABI1, PP2CA, and other clade A PP2Cs on endogenous SnRK1 signaling, we treated detached Arabidopsis leaves of the wild type, the double abi1-2 pp2ca-1 (Rubio et al., 2009), and two different quadruple pp2c knockout mutants (hai1-1 pp2ca-1 hab1-1 abi1-2, hereafter Qhai1-1; abi2-2 pp2ca-1 hab1-1 abi1-2, hereafter Qabi2-2; see Supplemental Figure 5 online; Antoni et al., 2013) under control (3 h of light [L]), activating (3 h of darkness [D]) and inactivating conditions (3 h of darkness followed by 1 h of darkness in 50 mM Glc [DG]), and analyzed SnRK1 target gene expression (Baena-González et al., 2007) by quantitative RT-PCR (qRT-PCR). Exposure to darkness triggered a strong induction of SnRK1 target genes in all genotypes (Figure 3C), in agreement with the current view that the conformation adopted by AMPK and Snf1 under conditions of low energy renders the kinases resistant to phosphatase action (Mayer et al., 2011; Oakhill et al., 2011; Xiao et al., 2011). In marked contrast, SnRK1 inactivation in response to subsequent Glc addition was deficient in abi1-2 pp2ca-1 plants

(for DIN6) and completely blocked in the quadruple pp2c mutants (Figure 3C), demonstrating that clade A PP2Cs are essential components for the post-stress inactivation of SnRK1 signaling.

In agreement with previous work (Baena-González et al., 2007), and despite the clear effect of PP2Cs on SnRK1 signaling under L, D, and DG conditions, analyses of total protein extracts of wild type and Qabi2 leaves revealed no clear differences with regard to T175 phosphorylation or total SnRK1 activity (Figures 3D and 3E). This suggests that subtle changes in SnRK1 phosphorylation and activity are sufficient to trigger significant downstream effects in gene expression, and that neither immunodetection with phospho-AMPKα (T172) antibodies nor SnRK1 kinase assays on total cellular SnRK1 are sensitive enough to monitor these changes.

Figure 3. ABI1 and PP2CA Repress SnRK1 Signaling.



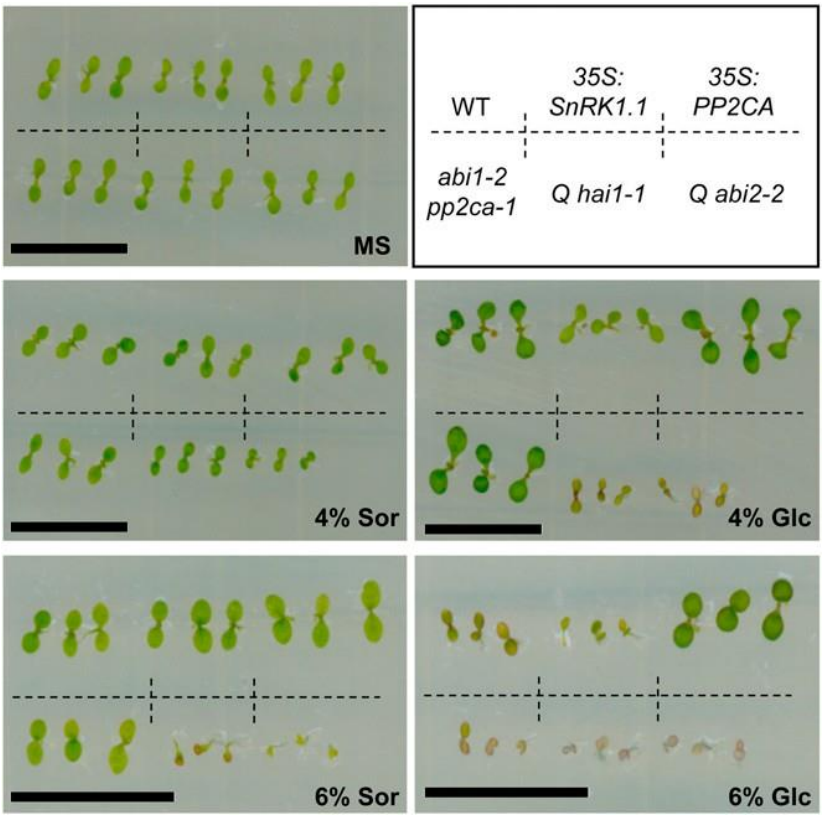
(A) SnRK1.1 activity, measured as the induction of the DIN6:LUC reporter in protoplasts is severely reduced by clade A PP2Cs ABI1 and PP2CA, but to a much lesser extent by the corresponding catalytically inactive mutants ABI1_D177A and PP2CA_D142A (n = 9). Numbers

above columns designate the percentage of SnRK1.1 inhibition as compared with 100% activity in the absence of PP2Cs **(B)** An unrelated clade E PP2C6-6 does not impinge on SnRK1.1 activity (n =8). **(C)** Reduced SnRK1 inactivation in double and quadruple pp2c knockout mutants Qhai1-1 and Qabi2-2. Relative gene expression of SnRK1.1 marker genes (DIN6, AXP) in control (L), activating (D), and inactivating (DG) conditions (n = 4). P values, one-way ANOVA with Tukey (A) and (B) and two-way ANOVA with Sidak test (C). Error bars = SE. Analyses of SnRK1(T175) phosphorylation (D) and SnRK1 activity (E) from total cellular extracts reveal no differences in various conditions and between wild-type and Qabi2-2 mutant plants. **(D)** SnRK1.1(T175) phosphorylation was detected by immunodetection with anti-phospho-AMPKα (T172) antibodies at the indicated time points. **(E)** SnRK1 activity was measured using SnRK1 immunoprecipitated from wild-type or Qabi2-2 leaves using the AMARA peptide assay. Values represent means 6 SD (n = 2).

Altered Sugar Responses in *pp2c* mutants

High concentrations of sugars (6% Glc; 330 mM) induce a developmental arrest characterized, for instance, by repression of cotyledon greening and expansion (Rolland et al., 2006). Wild type seedlings grow well on plates containing 4% Glc but cotyledon greening and expansion are clearly impaired on higher sugar concentrations (Figure 4). Such adverse conditions trigger SnRK1 activation, leading to sugar hypersensitivity in 35S:SnRK1.1 seedlings (Jossier et al., 2009) (Figure 4). The abi1-2 pp2ca-1 double mutant displays Glc hypersensitivity visible only in 6% Glc, but this is markedly enhanced in the quadruple pp2c mutants, which exhibit a clear phenotype in 4% Glc (Figure 4). Even though the ABA hypersensitivity of these mutants (Supplemental Figure 5) renders them more sensitive to increased osmolarity in the 4% sorbitol control plates (Antoni et al., 2012), a clear impact on development can be observed on 4% Glc plates. In 6% sorbitol and Glc plates, the growth of these mutants is so compromised that a distinction between osmotic and sugar effects is not possible. Consistent with the loss-of-function phenotype, plants overexpressing PP2CA are sugar insensitive (Figure 4), altogether genetically supporting the role of PP2Cs as negative regulators of SnRK1 signaling.

Figure 4. Altered Glc Response in *pp2c* knockout mutants and PP2C overexpressors.



Glc hypersensitivity of SnRK1.1 overexpressors (35S:SnRK1.1; 4-6% glc), double (abi1-2 pp2ca-1; 6% glc) and quadruple pp2c knockout mutants (Qhai1-1 and Qabi2-2; 4% glc), and Glc insensitivity of PP2CA overexpressors (35S:PP2CA; 6% glc) in early seedling development. Sor, sorbitol osmotic control; MS, control media without Glc or sorbitol. Bar = 1 cm.

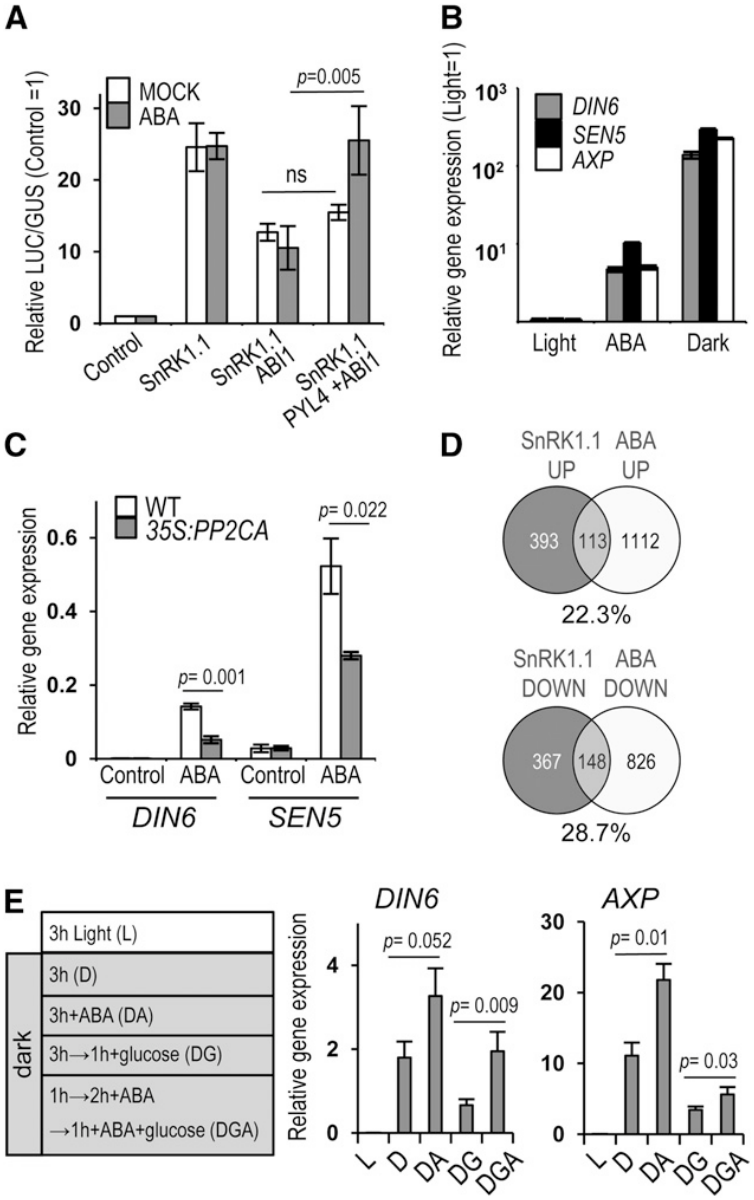
ABA Promotes SnRK1 Signaling via PP2Cs

We next wanted to assess whether PP2C regulation of the SnRK1 pathway could allow ABA to modulate SnRK1 activity. The transient coexpression of PYL receptors with ABI1 in ABA-treated mesophyll protoplasts is enough to efficiently repress ABI1 action and to trigger the activation of an ABA signaling reporter (Fujii et al., 2009). Similarly, coexpression of ABI1 with PYL4 in the presence of ABA fully restored SnRK1.1 ability to induce the DIN6: LUC reporter in protoplasts (Figure 5A), presumably through ABI1 sequestration in the ABA-PYL-PP2C ternary complex. We observed an overall twofold increase in LUC activity when comparing mock and ABA-treated samples (see Supplemental Figure 6 online), further suggesting that ABA can induce SnRK1 signaling. To further explore this possibility and to examine the effect of ABA on other SnRK1 target genes (Baena-González et al., 2007), we treated Arabidopsis leaf discs with or without ABA (100 μM) for 5 h and quantified downstream gene expression changes by qualitative qRT-PCR. ABA treatment did activate SnRK1, albeit to an extent 1 order of magnitude lower than that triggered by darkness (Figure 5B). Most importantly, the impact of ABA on SnRK1 target genes was reduced in plants overexpressing PP2CA (35S:PP2CA; Figure 5C) (Antoni et al., 2012), indicating that the effect of ABA on SnRK1 activity is via PP2C inhibition. To investigate this connection at the whole genome level, we compared the transcriptional profile associated with SnRK1.1 activation in protoplasts (Baena-González et al., 2007) with that of seedlings treated with ABA http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp, AtGenExpress Consortium; Nemhauser et al., 2006). Despite differences in tissue type and developmental stage in the two data sets, there was a significant overlap between the transcriptional changes triggered by SnRK1.1 and by ABA (Figure 5D). More than 22 and 28% of the total number of genes upregulated and downregulated by SnRK1.1, respectively, were similarly regulated by ABA, in marked contrast with the negligible overlap with other hormone treatments or when comparing genes oppositely regulated in the SnRK1.1 and ABA data sets (Supplemental Figure 7). Despite the wide impact of both SnRK1 and ABA on the transcriptome, the probability of

obtaining such an overlap of similarly regulated genes by chance is very low (hypergeometric test, $P < 9.2^{-42}$).

We next analyzed SnRK1 target gene expression in wild-type leaf discs subjected to ABA at the beginning of the dark treatment to test the combined effect of ABA and energy stress or 2 h prior to Glc addition to test the impact of ABA on the sugar-induced inactivation of SnRK1. Addition of ABA enhanced SnRK1 activation by darkness (Figure 5E, samples D, and DA). Moreover, adding ABA prior to Glc diminished SnRK1 inactivation in response to sugar (Figure 5E, samples DG and DGA). Collectively, these results show that ABA positively regulates SnRK1 signaling by inhibiting clade A PP2Cs, thereby promoting SnRK1 signaling during stress and once energy deficiency remits.

Figure 5. ABA Promotes SnRK1 signaling.



(A) PP2C repression of SnRK1 signaling in protoplasts is blocked by coexpression of the PYL4 receptor in the presence of ABA (n = 3). **(B)** Induction of SnRK1 target genes by ABA (n = 10) and energy stress (D; n = 12). **(C)** Reduced induction of SnRK1 target genes by ABA in 35S:PP2CA plants (n = 3). **(D)** SnRK1 activation and ABA treatment induce largely overlapping transcriptional responses. Percentage of upregulated or downregulated SnRK1.1 targets similarly regulated by

ABA. **(E)** ABA enhances SnRK1 activation by darkness and diminishes its Glc-triggered inactivation. SnRK1 target gene expression in L, DA, or D. Following dark activation, SnRK1 repression triggered by Glc was examined with (DGA) or without (DG) ABA pretreatment (n = 4). Error bars = SE. P values, two-way ANOVA with Fisher's least significant difference test. DIN6, SEN5, AXP, SnRK1, target genes.

DISCUSSION

Despite the central role of SnRK1 kinases in the plant stress response, the regulatory mechanisms underlying SnRK1 function are poorly understood. We have demonstrated here that ABI1 and PP2CA are bona fide SnRK1 phosphatases that contribute to resetting SnRK1 activity upon restoration of energy levels and that allow ABA to induce and potentiate SnRK1 signaling during stress (Figure 6). Although our results indicate that several clade A PP2Cs, including ABI1 and PP2CA, are important for SnRK1 regulation, this may not be true for all members of this clade. Furthermore, even though clade E PP2C6-6 had no significant impact on SnRK1 phosphorylation and signaling (Figures 2E and 3B), we cannot exclude the possibility that other PP2Cs regulate SnRK1 in other tissues or under different conditions. A clear interaction of SnRK1.1 with ABI1 and PP2CA was observed both in vitro and in vivo (Figure 1), demonstrating that PP2Cs act through direct binding to the SnRK1 *α*-catalytic subunit, probably using the C-terminal RD of SnRK1 as a docking site, albeit interacting also with the catalytic region that harbors the T175 target residue. Based on Y2H experiments, the KA1 domain of SnRK1 may play a key role in the PP2C–SnRK1 interaction (Figure 1A). As previously noted (Sánchez-Barrena et al., 2007), the KA1 domain can be closely superimposed on the phosphatase interaction domain of SOS2/SnRK3.11 and, given its presence also in the related AMPK and microtubule-affinity regulating kinase 3 kinases, has been suggested to represent an ancient highly conserved scaffold for interaction with PP2Cs (Sánchez-Barrena et al., 2007) (see Supplemental Figure 2 online). SnRK2.2/2.3/2.6 also require their C-terminal region, namely the ABA box, for PP2C binding (Vlad et al., 2009; Soon et al., 2012), and additional regions of interaction exist within the N-terminal CD (Soon et al., 2012), some of which, such as the T-loop and the *α*G helix, correspond to conserved features of the protein kinase canonical fold (Hanks and Hunter, 1995) (see Supplemental Figure 2 online). Our in vitro pull-down assays suggested that the SnRK1.1-PP2CA interaction may not solely rely on the SnRK1 RD and that similarly to SnRK2s, some parts of the CD may also play a role in this interaction (Figure 1B). Interestingly, a high-throughput screen for YFP-ABI1 interactors employing

affinity purification and liquid chromatography coupled with tandem mass spectrometry identified SnRK1s as candidate ABI1-interacting proteins, whereas peptides corresponding to SnRK2.6 were not retrieved and the ABI1–SnRK2.6 interaction could only be confirmed by co-immunoprecipitation of the transiently overexpressed proteins in tobacco (*Nicotiana benthamiana*) (Nishimura et al., 2010).

As an outcome of the interaction with ABI1 and PP2CA, SnRK1 is dephosphorylated and inactivated (Figures 2 and 3). Nevertheless, disruption of the catalytic site in the ABI1_D177A and PP2CA_D142A mutants did not fully restore SnRK1 activity (Figure 3A), suggesting that, although dephosphorylation plays a major role in SnRK1 inactivation, physical blockage may, similarly to SnRK2s (Soon et al., 2012), also be important for SnRK1 repression. The mechanism of action also may differ between the various PP2Cs, as suggested by the fact that despite having a lower impact on SnRK1 (T175) phosphorylation (Figure 2E), PP2CA had a consistently stronger effect than ABI1 on SnRK1 signaling (Figure 3A). Given that the SnRK1 RD is the major region of interaction with PP2Cs (Figure 1) and that this region is responsible for binding the regulatory subunits (Bhalerao et al., 1999; Kleinow et al., 2000), it is plausible that PP2C binding affects SnRK1 activity also by interfering with trimer formation.

PP2CA was able to efficiently dephosphorylate T175 in vitro and in vivo (Figure 2), consistent with the in vitro dephosphorylation of this residue by mammalian PP2C (Sugden et al., 1999). Nevertheless, despite the clear differences in gene expression observed between control, inducing, and inactivating conditions and between the wild-type and Qabi2-2 leaves (Figure 3C), we were unable to detect differences in T175 phosphorylation or SnRK1 activity in these conditions in the endogenous SnRK1 (Figures 3D and 3E), suggesting that the relatively short treatment times employed result in subtle changes in kinase phosphorylation and activity that are not possible to detect with the phospho-AMPKa(T172) antibodies or the kinase activity assays from total cellular SnRK1.1. Indeed, a much longer (24-h) starvation treatment of rice suspension cells resulted in mild (1.9 fold) differences in SnRK1 activity, as measured

with the SAMS peptide (Lu et al., 2007). These results are in agreement with the view on cellular enzyme cascades in which slight changes in enzyme activity may trigger significant downstream effects by amplifying the signal (Chock et al., 1980). More sensitive and quantitative techniques like Mass Western (Lehmann et al., 2008) and/or the enrichment of specific SnRK1 subcellular pools may be required for accurately assessing changes in SnRK1 T-loop phosphorylation and activity in response to stress and nutrient signals.

Our results employing reporter gene assays and gene expression analyses in the wild type, pp2c knockout mutants, and PP2CA overexpressors show that PP2Cs are negative regulators of SnRK1 signaling (Figures 3 and 4). Transient coexpression of ABI1 and PP2CA with SnRK1 in protoplasts reduced by 60% the ability of SnRK1 to activate gene expression (Figure 3). Using a similar approach, Fujii and colleagues showed that the extent of repression by ABI1 was nearly 100% when coexpressing SnRK2.6 and its downstream ABF2 transcription factor to activate an ABA reporter (Fujii et al., 2009). However, the ability of PP2Cs to repress kinase activity varied depending on the SnRK2 and PP2C combination employed, and in the case of SnRK2.6 and HAB1, the repression was only 30%. Because some clade A PP2Cs have been shown to dephosphorylate ABF2 (Antoni et al., 2012), it is also possible that the difference in the extent of repression is due to a simultaneous effect of ABI1 on the kinase and on the transcription factor.

Most importantly, constitutive PP2C depletion in the quadruple *pp2c* mutants abrogates SnRK1 inactivation and downstream target gene repression after stress-derived energy deprivation subsides (Figure 3C, DG samples). However, the impact of PP2C depletion is less obvious under activating stress conditions (Figure 3C, D samples) presumably because, as for AMPK and Snf1 (Mayer et al., 2011; Oakhill et al., 2011; Xiao et al., 2011), the kinase is protected from dephosphorylation when energy levels are low (Sugden et al., 1999). Similarly to plants overexpressing SnRK1.1, double and quadruple pp2c knockout mutants showed varying degrees of a sugar hypersensitive

phenotype, while PP2CA overexpressors displayed an opposite phenotype (Figure 4), all consistent with the conclusions from the molecular data that PP2Cs negatively regulate SnRK1.

Our results indicate that the ABA and energy signaling pathways interact through PP2Cs and that ABA can induce SnRK1 signaling through PP2C inhibition (Figure 5). This is in agreement with a recent study reporting enhanced SnRK1 activity in wheat (*Triticum aestivum*) roots in response to ABA (Coello et al., 2012), and provides a molecular explanation for the extensive interactions observed between ABA and sugar signaling in genetic screens (Rolland et al., 2006). SnRK1s were never identified among ABA-activated kinases, most probably because the extent of SnRK1 activation by ABA is 1 order of magnitude lower than that by energy stress (Darkness; Figure 5B), and would probably remain masked by the much stronger activities of SnRK2s. In contrast, these studies relied on in-gel kinase assays for detecting of kinase activities (Yoshida et al., 2002; Furihata et al., 2006; Fujii et al., 2007). Despite our current lack of knowledge regarding the exact subunit composition of functional SnRK1, and despite the fact that the catalytic subunit alone is active (Bhalerao et al., 1999; Shen et al., 2009; Crozet et al., 2010), in vivo SnRK1 most likely operates, similarly to Snf1 and AMPK, as a heterotrimeric complex (Polge and Thomas, 2007; Hedbacker and Carlson, 2008; Hardie, 2011; Ramon et al., 2013), whose dissociation under the denaturing conditions employed in the in-gel kinase assays may result in loss of kinase activity.

In addition to the interaction through PP2Cs, other points of crosstalk are likely to exist between ABA and energy signaling, and SnRK1 may regulate ABA transcription factors, such as ABF2 (Figure 2B) or FUS3 (Zhang et al., 2008; Tsai and Gazzarrini, 2012) that can also be directly dephosphorylated by PP2Cs (Antoni et al., 2012). It is conceivable that aberrant PP2C:SnRK1 ratios as well as the possible PP2C/SnRK1 coregulation of downstream factors could account for the altered ABA sensitivity and ABA- related phenotypes of plants with altered SnRK1 signaling (Radchuk et al., 2006; Lu et al., 2007;

Rosnoblet et al., 2007; Jossier et al., 2009; Radchuk et al., 2010; Tsai and Gazzarrini, 2012).

We propose a dual role for the regulation of SnRK1 by ABI1 and PP2CA (Figure 6). On one hand, activation of the SnRK1 pathway through alternative signals like ABA, could support the ABA response with a more general one directed toward a metabolic and transcriptional reprogramming to cope with energy deficiency. Activation of SnRK1 by ABA could also serve to prime the SnRK1 system, potentiating a subsequent response to energy imbalance derived from stress. On the other hand, PP2C regulation appears to be an integral part of the SnRK1 signaling pathway, resetting the system once stress subsides or an energy balance is attained through the appropriate metabolic readjustments. Persistence of ABA under these conditions would in turn promote the maintenance of SnRK1 in an active state, similarly to how elevated interleukin-6 sustains high AMPK activity in skeletal muscle when energy levels are presumably no longer altered after exercise (Ruderman et al., 2006). With this scenario in mind, one could envision that in tissues directly exposed to stress, SnRK1 activation would be mainly dictated by the energy-dependent branch, whereas in distant tissues, this activation could be mediated by ABA. In addition to interleukin-6, AMPK responds to other inflammatory mediators and hormones, but the precise mechanisms underlying this regulation are in most cases unknown (Steinberg and Kemp, 2009; Lim et al., 2010). Interestingly, chronic Tumor Necrosis Factor a treatment in muscle cells suppresses the AMPK pathway by inducing the repressor PP2C (Steinberg et al., 2006), suggesting that a connection between hormone signals and energy signaling through the inhibitory PP2Cs might be conserved in multicellular eukaryotes.

In summary, we have identified ABI1 and PP2CA as upstream phosphatases of SnRK1, uncovering a mechanism through which ABA can stimulate SnRK1 action. Future work to further understand SnRK1 regulation and to unravel the interplay of these two central pathways may offer insight not only into the mechanisms of stress tolerance

but also into fundamental developmental processes, such as seed maturation and germination.

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Supplementary material

Supplementary Figure 1

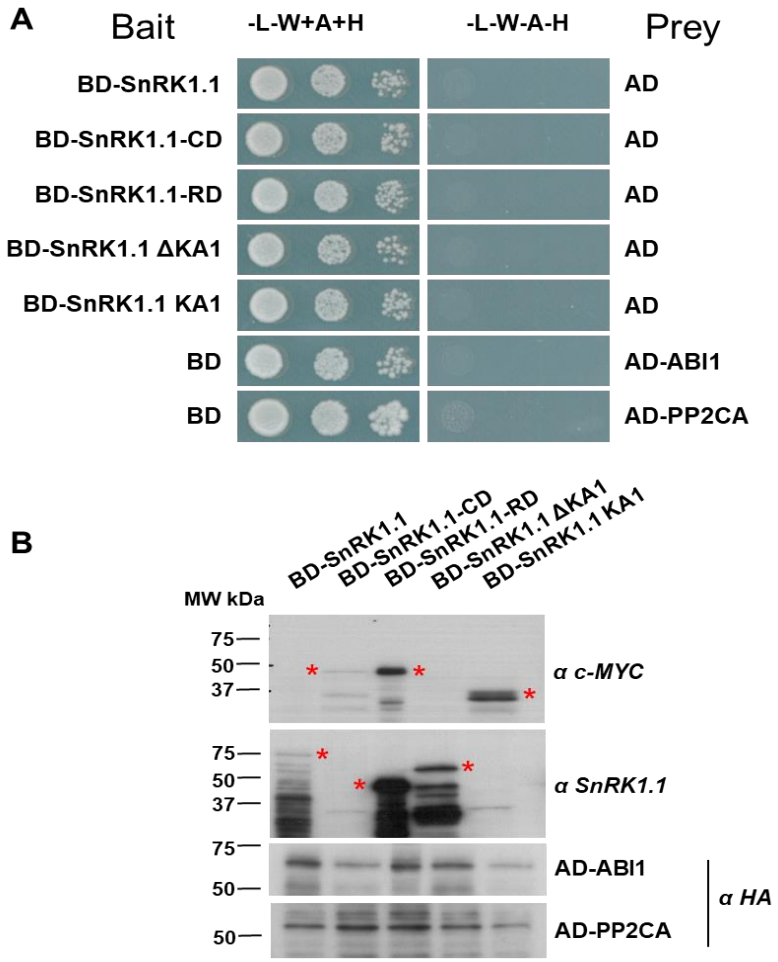


Figure S1. Yeast-two-hybrid controls for the SnRK1.1 and PP2C interaction (Fig. 2*A*). **(A)** None of the AD and BD constructs activate the *ADE* and *HIS* reporters. Colony growth was assessed on medium lacking adenine and histidine (-A-H) using serial dilutions (10-1, 10-2, and 10-3) of saturated cultures. The different SnRK1.1 deletions are shown. CD=catalytic domain, residues 1-293; RD=regulatory domain, residues 294-512; KA1 domain=residues 390-512. AD=GAL4

activation domain, BD=GAL4 binding domain. **(B)** Expression of the indicated constructs in yeast as revealed by immunodetection with anti-HA (for AD-constructs) and anti-c-MYC (for BD-constructs) antibodies. Full-length SnRK1.1 and SnRK1.1 ΔKA1 have low expression levels and are more readily detected with the anti-SnRK1.1 antibody. Note that this antibody is against a peptide in the more proximal part of the RD-region and thus does not detect SnRK1.1-CD nor SnRK1.1 KA1. Red asterisks indicate the band with the expected molecular weight.

Supplementary Figure 2

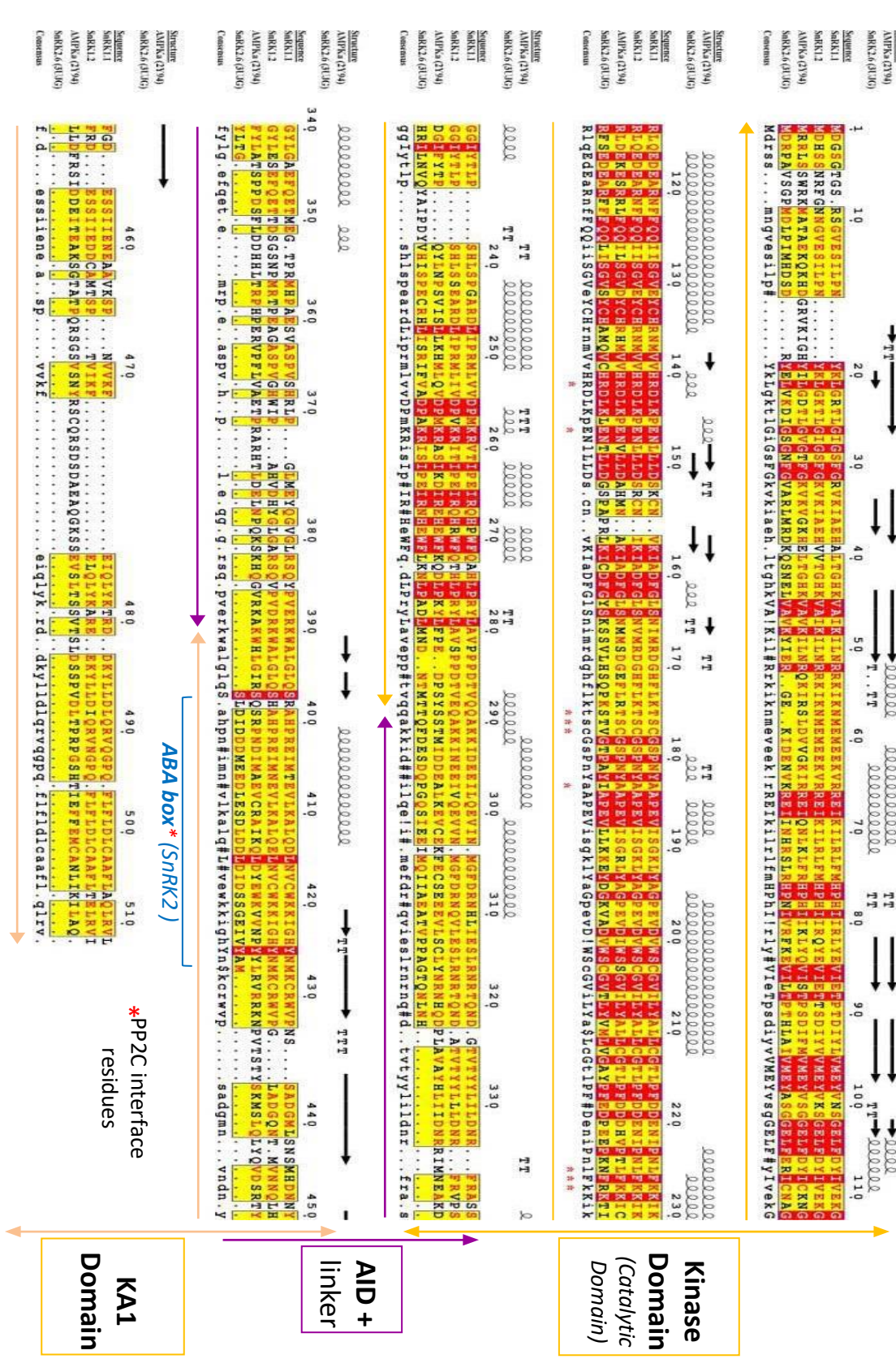


Figure S2. Alignment and structural comparison of SnRK1 and SnRK2. **(A)** Alignment of SnRK1.1 (Q38997), SnRK1.2 (P92958), AMPKα (PDB: 2Y94-A) and SnRK2.6 (PDB: 3UJG-A) was performed with ClustalW and represented with ESPript (Gouet *et al.*, 1999), displaying the known secondary structures on the top. Residues fully conserved in all four sequences are in red and those conserved in three in yellow. Residues marked by a red asterisk are implicated in physical interaction with the HAB1 PP2C phosphatase (3UJG) (Soon *et al.*, 2012). Kinase Domain (KD, catalytic domain, CD; common to the four proteins) is marked by orange arrows and the KA1 domain (only for SnRK1 and AMPK) in marked by blue arrows. "AID + linker" (marked by purple arrows) stands for "Auto-Inhibitory Domain" followed by a linker region by analogy with the AMPKα (Hardie *et al.*, 2012). No function has been assigned to this sub-domain in plants.

Supplementary Figure 2

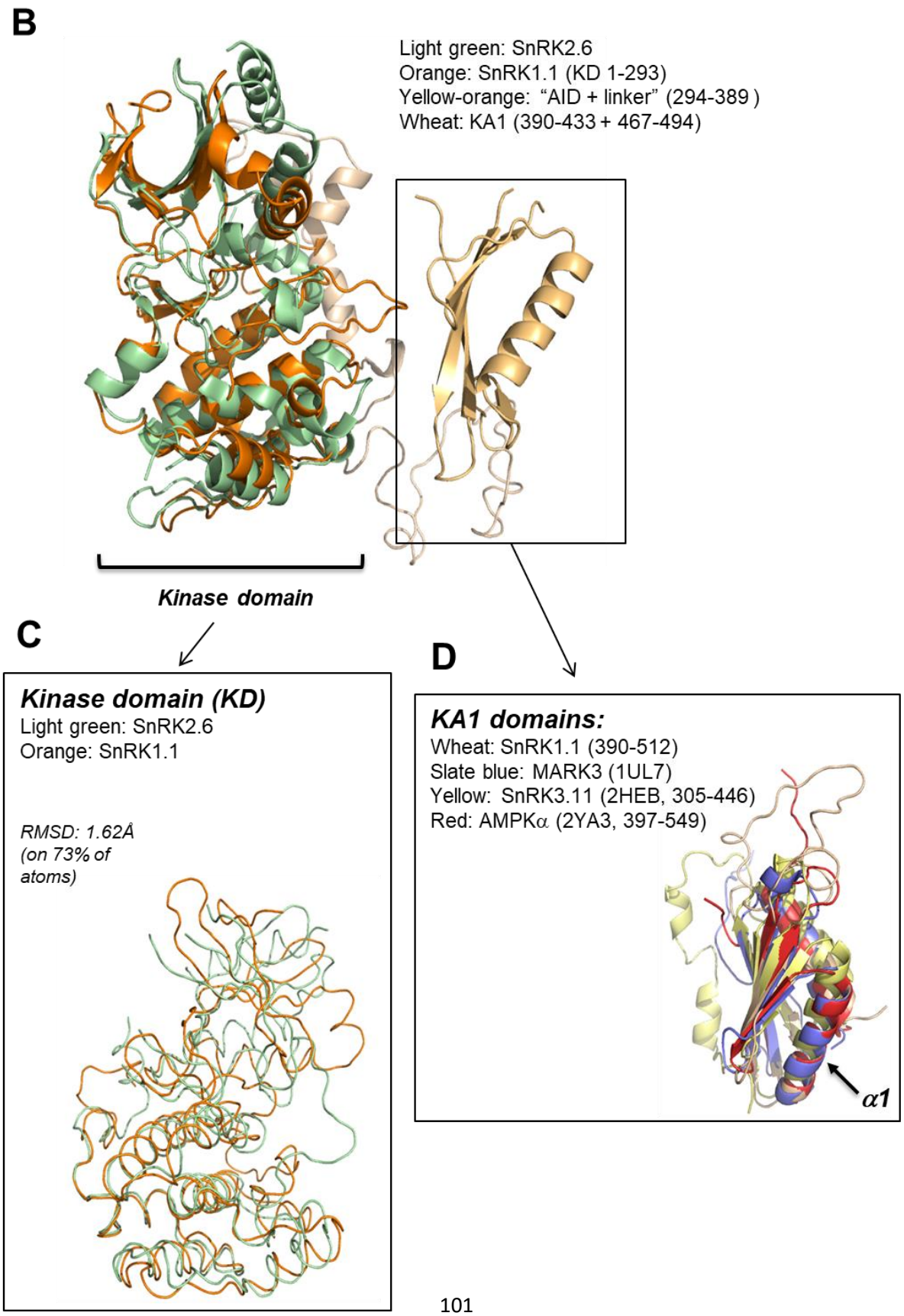


Figure S2. Alignment and structural comparison of SnRK1 and SnRK2 **(B)** Structural alignment of the SnRK1.1 model [performed from template 2Y94S (Xiao *et al.*, 2011) with Swiss-model (Arnold *et al.*, 2006)] with SnRK2.6 (3UJG-A). Colored as described, cartoon representation. **(C)** Structural alignment of the kinase domain of SnRK1.1 model with SnRK2.6. RMSD of kinase domain alignment is 1.62Å on 73% of aligned atoms, giving confidence on the conservation observed in alignment (see **A**). As almost all the important residues (* in **A**) are in loops, no more can be assessed for these. The other three are located in the αG helix of the kinase domain in its large lobe (subdomain XS) (Hanks & Hunter, 1995). The large lobe alignment of these kinases is good (RMSD=0.81Å on 74% of aligned atoms) giving confidence in these conservation. Colored as described, ribbon representation. **(D)** Validation of the Kinase Associated1 (KA1) domain model of SnRK1.1. KA1 domain from Uniprot database is annotated as shorter (486-512) than our considered model (390-512). Comparison of the actual structures of a SnRK3.11/SOS2 (2HEB) (Sánchez-Barrena *et al.*, 2007), MARK3 (1UL7) (Tochio *et al.*, 2006), the AMPKα "core complex" part (2YA3) (Xiao *et al.*, 2011) with a model of the last 122 residues of SnRK1.1 (part colored blue in **(A)** modeled by Phyre (Kelley & Stenberg, 2009). This part is clearly exhibiting a KA1 fold with a β-sheet (of four β-strands) and two α-helixes on the same side of the β-sheet. Colored as stated, cartoon representation. All images and structural alignment were generated with Pymol (from Delano Scientific). α1 refers to the α-helix part of the phosphatase interacting domain (PPI) (Sánchez-Barrena *et al.*, 2007).

Supplementary Figure 3

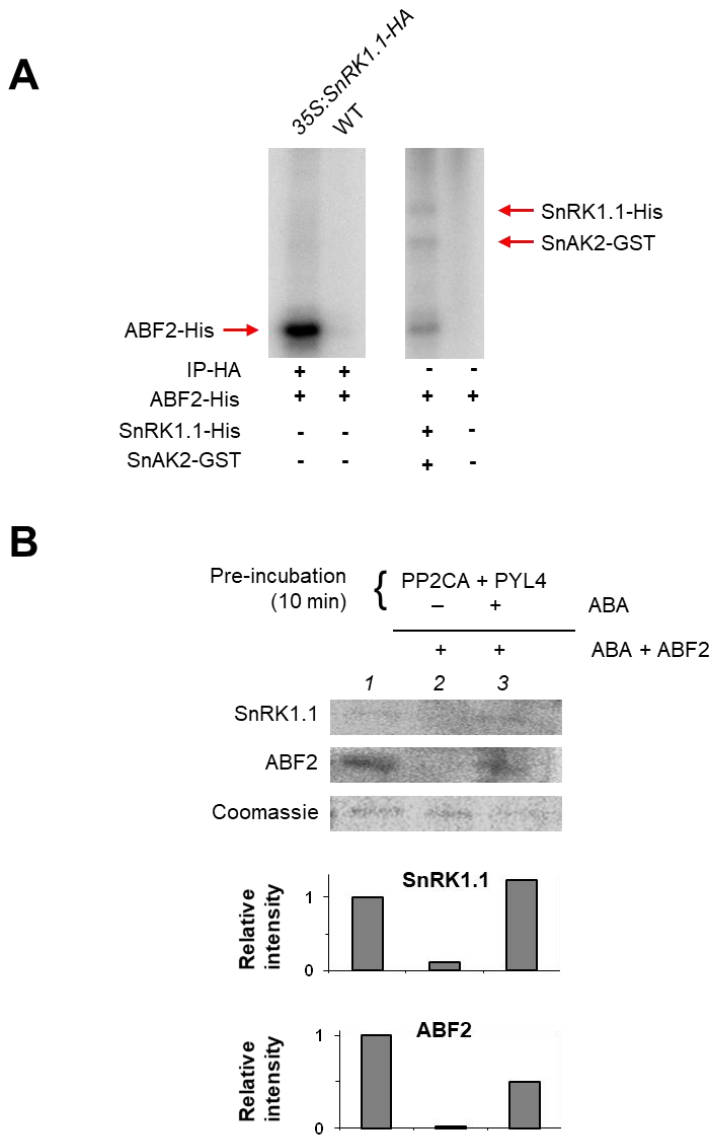


Figure S3. SnRK1.1 is inactivated by recombinant His-PP2CA *in vitro*. **(A)** Control HA-immunoprecipitation from WT plants retrieves no ABF2 phosphorylating activity, showing that the activity measured from *35:SnRK1.1-HA* plants is specific to SnRK1.1. Right panel, positive control showing that recombinant SnRK1-His preactivated with SnAK2-GST phosphorylates ABF2. **(B)** Where indicated SnRK1.1 was pre-incubated, for 10 min, with PP2CA and PYL4 in the absence (lane 2) or presence (lane 3) of ABA, to allow or prevent PP2CA activity, respectively. After this pre-incubation ABA was added to all samples to inactivate PP2CA, the ABF2 substrate was supplied, and the reaction was further incubated for 1h.

Supplementary Figure 4

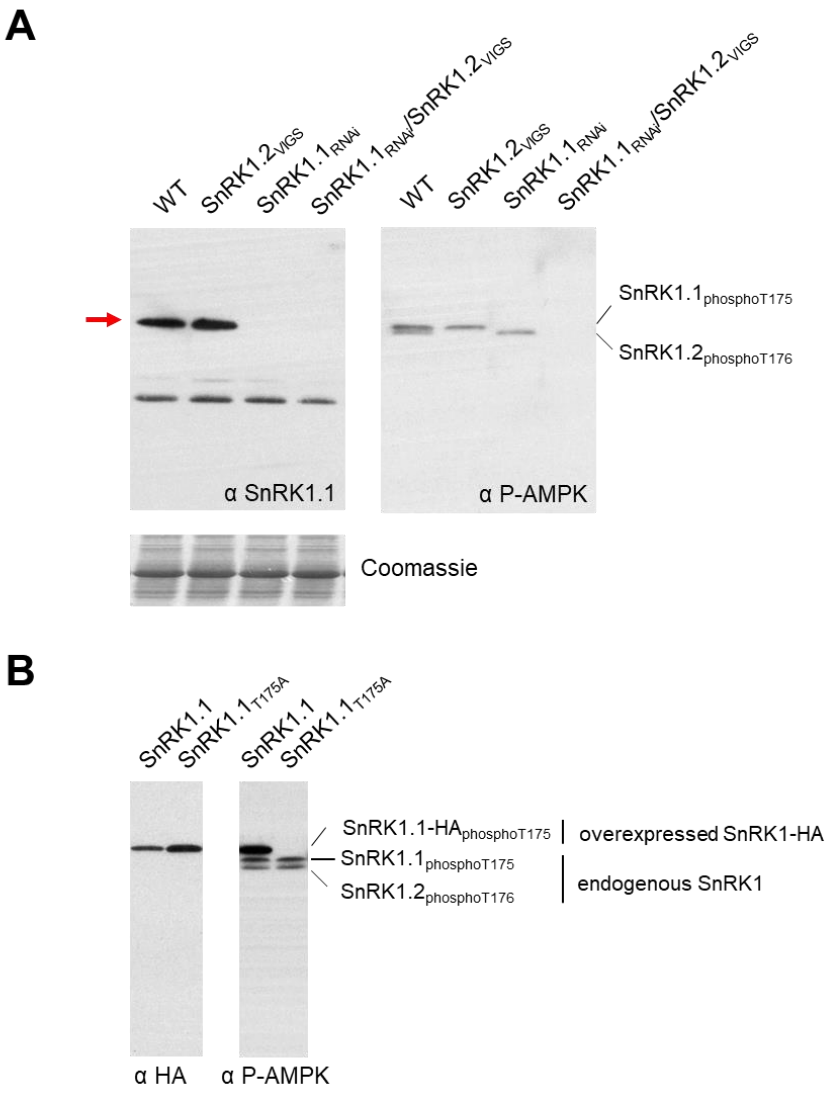


Figure S4. Specific detection of phosphorylated SnRK1. (A) The P-AMPK antibody recognizes specifically SnRK1.1 and SnRK1.2 in total protein extracts from *Arabidopsis* leaves. WT and SnRK1.1 RNAi plants were infiltrated with *Agrobacterium* containing viral vectors for a GFP control (WT) or for VIGS of SnRK1.2 and analyzed 3 weeks after, using anti-SnRK1.1 and anti-P-AMPK antibodies (Baena-González et al., 2007). The red arrow indicates the band corresponding to SnRK1.1. **(B)** Mutation of T175 to A abolishes SnRK1.1-HA recognition by the P-AMPK antibody. *Arabidopsis* mesophyll protoplasts were transfected with constructs expressing SnRK1.1-HA or SnRK1.1T175A-HA and proteins were detected after SDS-PAGE by immunoblotting with anti-HA or anti-P-AMPK antibodies.

Supplementary Figure 5

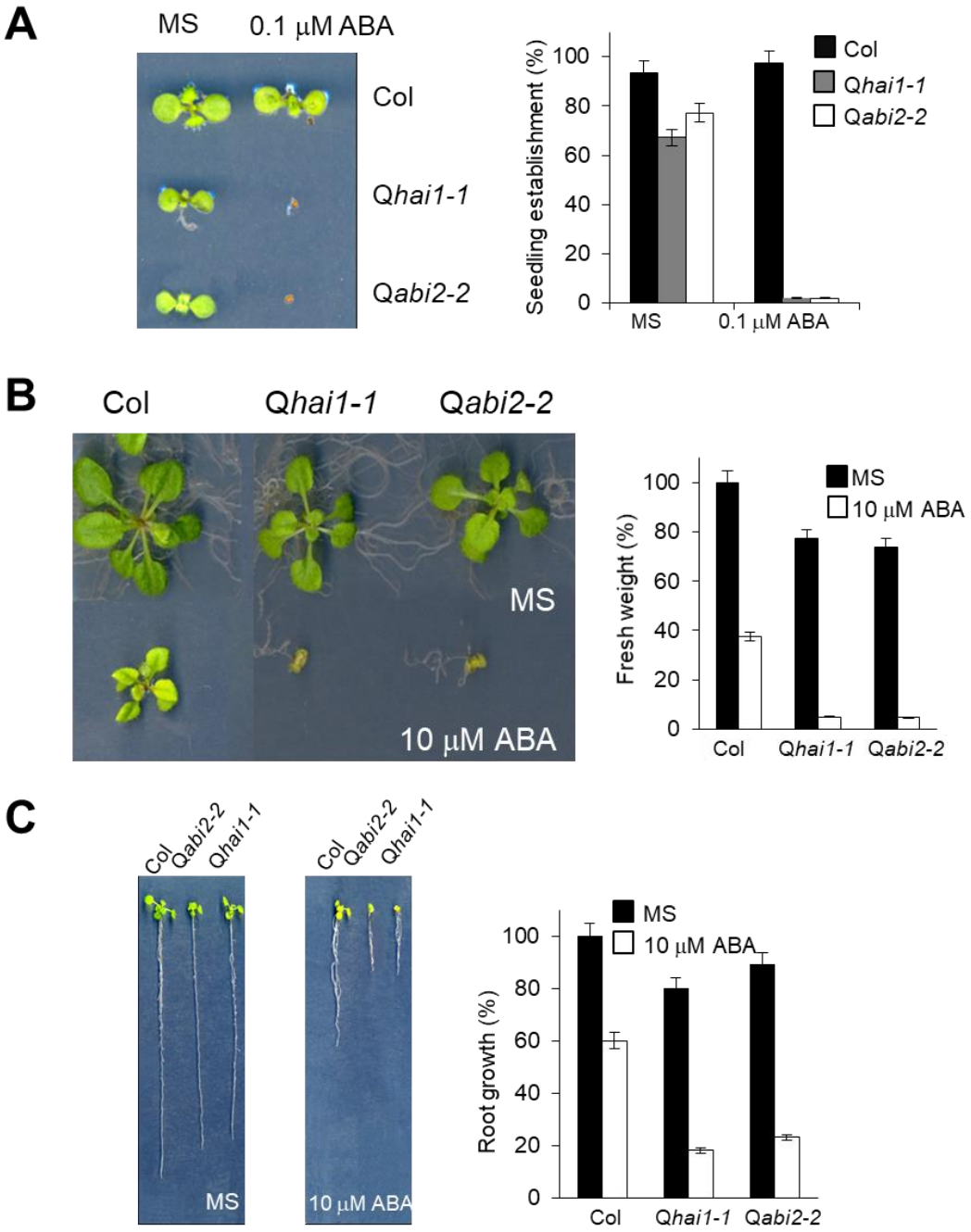


Figure S5. Clade A *pp2c* quadruple mutants are ABA–hypersensitive. **(A)** Enhanced sensitivity to inhibition of seedling establishment by ABA. Seeds were germinated and grown in medium lacking or supplemented with 0.1 µM ABA for 10 days (*n*=100). **(B)** The growth of the *pp2C* mutants is not strongly affected in control MS medium but is impaired in medium containing 10 µM ABA. Photographs were taken 20 days after transferring 5-day-old seedlings from MS

medium to plates lacking or containing 10 μM ABA (*n*=15). **(C)** ABA-hypersensitive root growth inhibition of *pp2c* mutants. Photographs were taken 10 days after transferring 4-day-old seedlings to MS plates lacking or supplemented with 10 μM ABA (*n*=15). *Col,* Columbia wild-type*;* Q*hai1-1, hab1-1 abi1-2 pp2ca-1 hai1-1*; Q*abi2-2, hab1-1 abi1-2 pp2ca-1 abi2-2* (Antoni *et al.,* 2013). Values represent means±SEM.

Supplementary Figure 6

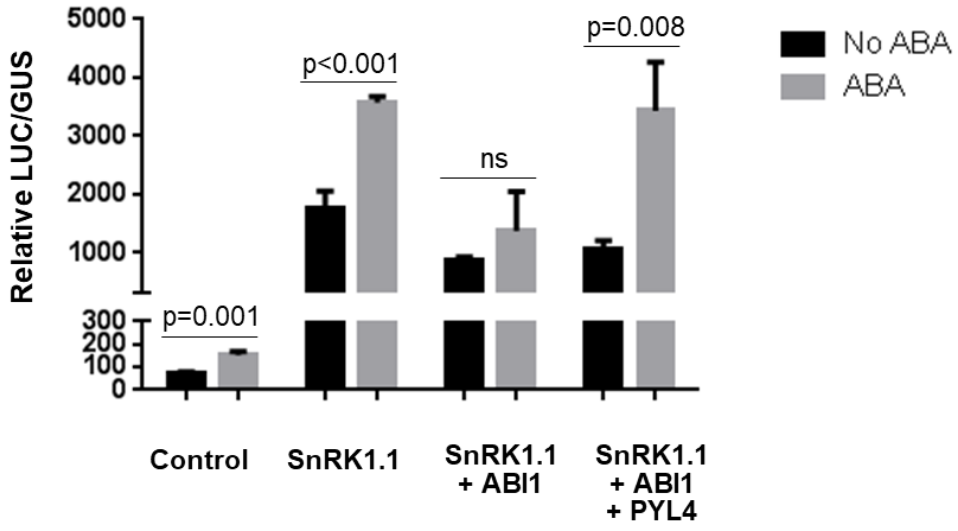


Figure S6. ABA promotes SnRK1 signaling in protoplasts. Cells were transfected with control DNA, or with plasmids expressing SnRK1.1 alone or in combination with ABI1 and the PYL4 receptor. In the absence of overexpressed PYL4, ABA and the endogenous receptors are not sufficient to inhibit overexpressed ABI1. Samples are the same as in Fig. 2A, but instead of normalizing the mock and ABA sets to their corresponding controls, all samples were normalized to the mock control (n=3). Values represent means±SEM. p-values, multiple t-test with Holm-Sidak correction.

Supplementary Figure 7

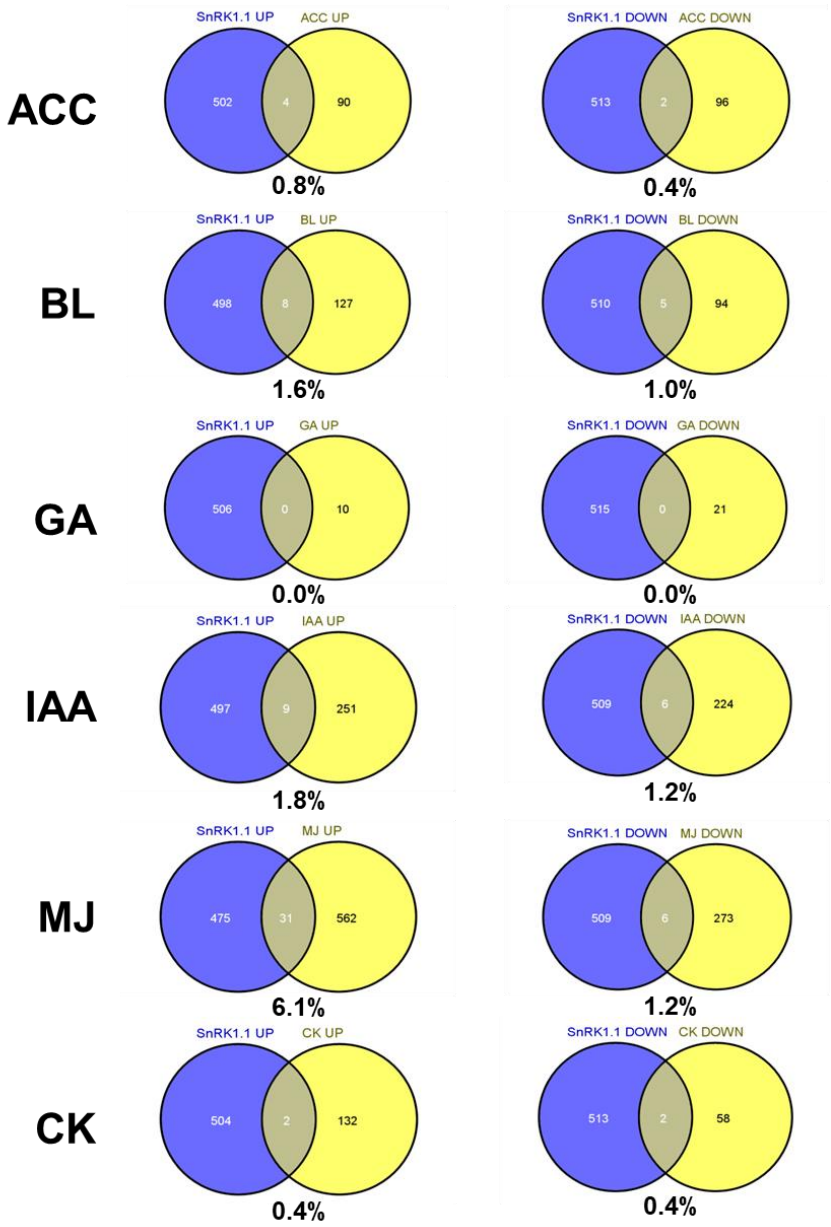


Figure S7 Overlap between transcriptional changes induced by SnRK1.1 (Baena-González, Rolland, *et al.*, 2007) and indicated hormone treatments (Nemhauser et al., 2006; AtGenExpress). UP and DOWN denote the set of up- or down-regulated genes, respectively, in the indicated datasets. Percentage values refer to the number of overlapping genes per total number of upregulated or downregulated SnRK1.1 targets. ACC, 1-aminocyclopropane-1-carboxylic acid (ethylene precursor); BL, brassinolide; GA, gibberellic acid; IAA, indole-3-acetic acid (auxin); MJ, methyl jasmonate; CK, cytokinin

Supplementary Figure 7 (cont.)

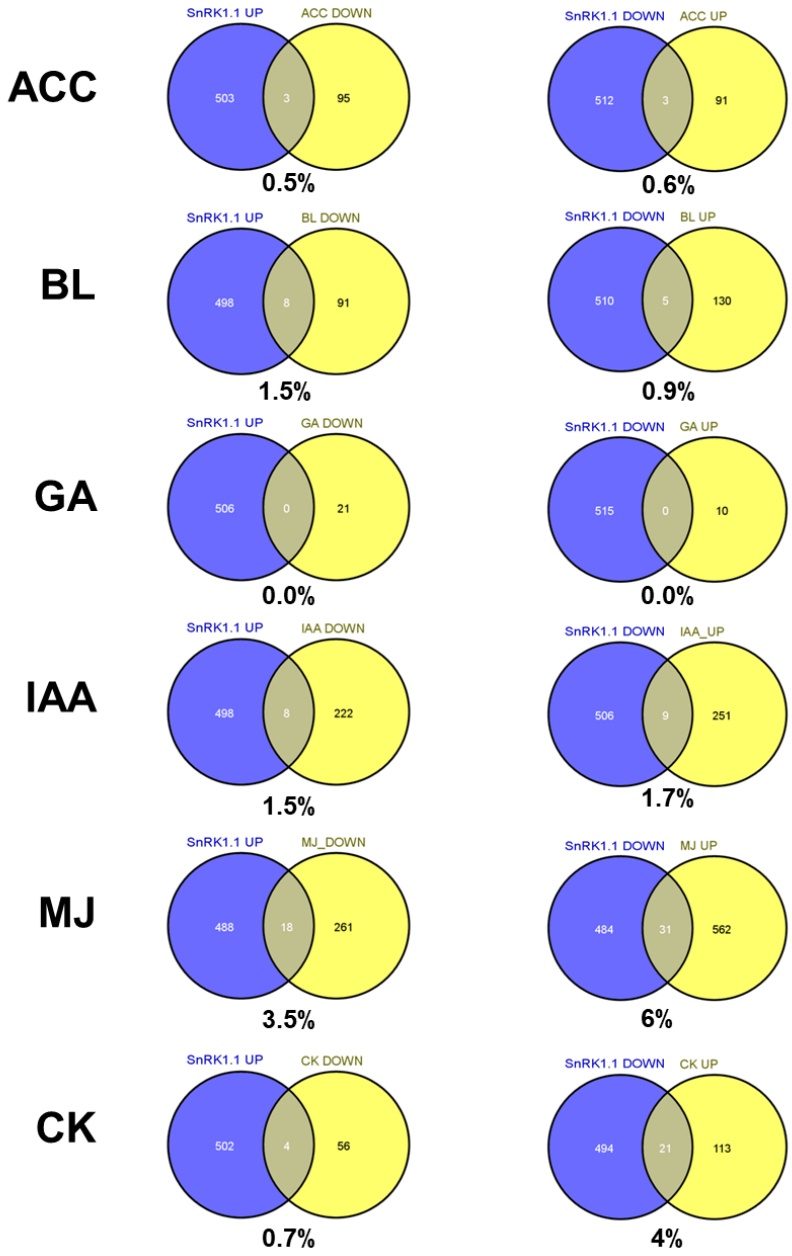
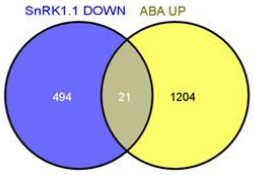


Figure S7 (cont.) Overlap between transcriptional changes induced by SnRK1.1 (Baena-González, Rolland, *et al.*, 2007) and indicated hormone treatments (Nemhauser et al., 2006; AtGenExpress). UP and DOWN denote the set of up- or down-regulated genes, respectively, in the indicated datasets. Percentage values refer to the number of overlapping genes per total number of upregulated or downregulated SnRK1.1 targets. ACC, 1-aminocyclopropane-1-carboxylic acid (ethylene precursor); BL, brassinolide; GA, gibberellic acid; IAA, indole-3-acetic acid (auxin); MJ, methyl jasmonate; CK, cytokinin

Supplementary Figure 7 (cont.)



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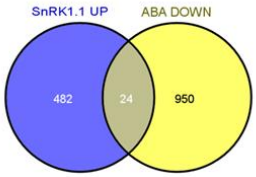

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Figure S7 (cont.) Overlap between transcriptional changes induced by SnRK1.1 (Baena-González, Rolland, *et al.*, 2007) and ABA (Nemhauser et al., 2006; AtGenExpress). Overlap between the genes induced by SnRK1.1 and repressed by ABA, and between the genes repressed by SnRK1.1 and induced by ABA. UP and DOWN denote the set of up- or down-regulated genes, respectively, in the indicated datasets. Percentage values refer to the number of overlapping genes per total number of upregulated or downregulated SnRK1.1 targets.

Material and methods

Plant Material and Growth Conditions

All used Arabidopsis thaliana plants are in the Columbia (Col-0) background, except 35S:SnRK1.1-HA (*Landsberg erecta*) (Baena-González et al., 2007). The 35S:SnRK1.1 (35S:SnRK1.1-2) (Jossier et al., 2009), 35S:PP2CA (Antoni et al., 2012), and abi1-2 pp2ca-1 (Rubio et al., 2009) lines have been described. Quadruple *pp2c* knockout mutants were generated from *pp2ca-1 hai1-1* (Antoni et al., 2012) and the corresponding triple *pp2c* mutants (Rubio et al., 2009). Plants were grown in soil under a 12-h-light (100 µE)/12-h-dark regime. For in vitro culture, sterilized seeds were stratified in the dark at 4°C for 2 days and sowed on plates containing Murashige and Skoog medium with 0.1% MES, 0.8% phytoagar, and Glc (4 or 6%) or sorbitol (4 or 6%). Plates were sealed and incubated at 23°C under continuous light.

Antibodies and Protein Expression Analyses

The SnRK1.1 antibody was purchased from Agrisera (anti-AKIN10, AS10919). Phospho-SnRK1.1(T175) was detected with an anti–phospho- AMPKα (T172) antibody (referred to as α P-AMPK; Cell Signaling), which also detects phospho-SnRK1.2(T176) as a lower band (Baena-González et al., 2007). An anti-GST polyclonal antibody (Sigma), anti-HA (Roche), and anti-T7 (Novagen) monoclonal antibodies were used to detect the corresponding tagged proteins. For analyses of protein expression from protoplast pellets and leaf tissue, the material was directly ground in 2x Laemmli solubilization buffer to maintain the phosphorylation status during protein extraction.

Protoplast Transient Expression Assays

Vectors for protoplast transient expression and assays were as described (Yoo et al., 2007), using the UBQ10-b-glucuronidase reporter as transfection efficiency control. For constructs for overexpression of SnRK1.1-GFP, ABI1- HA, PP2CA-HA, PP2C6-6-HA, and FLAG-PYL4, the corresponding coding sequences were cloned into a pHBT95 vector harboring the indicated C- or N-terminal tag. SnRK1 signaling was monitored using a DIN6:LUC reporter (Baena-González et al., 2007).

ABA and Glc were added to a final concentration of 5 µM and 30 mM, respectively. For coimmunoprecipitation assays, untagged SnRK1.1 was expressed with ABI1-HA or mER7 control DNA (Yoo et al., 2007) in 3 ml of protoplasts (6 * 10^5 cells) under standard conditions.

Frozen cell pellets were lysed in 500 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM EDTA, 10% glycerol, 0.5% Triton X-100, and complete protease inhibitor cocktail [Roche]), 20 mM sodium fluoride, 1 mM orthovanadate, 1/500 (v/v) phosphatase inhibitor 2 (Sigma P044), and 1/500 (v/v) phosphatase inhibitor 3 (Sigma P5726)], incubated at 4°C for 10 min, and diluted to a final volume of 1.5 ml with lysis buffer without Triton X-100. The cleared lysate was incubated with 40 µl of anti-HA affinity matrix (Roche 11815016001) for 3h at 4°C. Agarose beads containing immunoprecipitated proteins were washed five times with lysis buffer containing 0.05% Triton, eluted with 4x Laemmli solubilization buffer, and analyzed by immunoblotting with an anti-SnRK1.1 antibody.

Recombinant Protein Production

The coding sequence of PP2CA was cloned into pGEX-4T1. Recombinant GST-PP2CA was produced in Escherichia coli (BL21:DE3) and purified through S-linked glutathione agarose affinity chromatography as recommended by the manufacturer (Sigma G4510).

N- (residues 1 to 293, CD) and C-terminal (residues 294–512, RD) SnRK1.1 were cloned into pET28a (Novagen). Recombinant proteins were produced in E. coli (BL21:DE3) and purified using immobilized metal ion affinity chromatography (TALON, BD Clontech) following the manufacturer's instructions. Successful protein production and purification were verified by immunoblotting with anti-GST and anti-T7 antibodies. Recombinant His-PYL4, His-PP2CA, and His-DC ABF2 (residues 1–173) were produced as described by Antoni et al. (2012), and recombinant GST-SnRK1.1 and GST-SnAK2 were produced as described by Crozet et al. (2010).

In Vitro Pull-Down Assays

Proteins (3 µg of each) were incubated for 1 h at room temperature in 100 ml of buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% Triton X-100, and 1/500 [v/v] plant-specific protease inhibitor cocktail [Sigma P9599]), mixed with 30 ml of glutathione–agarose beads and incubated one more hour. Beads were washed four times with buffer A, and bound proteins were analyzed by immunoblotting using anti-T7 antibodies.

SnRK1.1 Immunoprecipitation, Phosphatase Treatment, and in Vitro Kinase Assays

SnRK1.1 was immunoprecipitated from leaves of 35S:SnRK1.1-HA plants treated for 1 h in darkness. Plant material (1 g) was extracted in 3 volumes of PBS supplemented with 1 mM EDTA, 0.05% Triton X-100, and 1/500 (v/v) plant-specific protease inhibitor cocktail (Sigma). After centrifugation (16,000g, 4°C, 15 min), the supernatant was recovered, and 1 µg of total protein was incubated overnight at 4°C with 30 µl of anti-HA affinity matrix. The matrix was washed three times with extraction buffer and resuspended in a total volume of 66 µl of buffer (50 mM Tris-HCl, pH 7.6, 250 mM KCl, 10% glycerol, and 0.1% Tween 20), of which 3 µl was used for each reaction.

To assess dephosphorylation of immunoprecipitated SnRK1.1 by PP2CA, SnRK1.1 was incubated with His-PP2CA (2 mg) in a 50 ml reaction containing 25 mM Tris-HCl, pH 7.5,

10 mM $MgCl_2$, and 1 mM DTT. The reaction was stopped with Laemmli solubilization buffer and analyzed by Phos-Tag SDS-PAGE (50 mM Phos-Tag ligand [Wako] and 100 mM $MnCl_2$) (Kinoshita et al., 2009) and immunoblot with an anti-HA antibody. The Phos-Tag ligand selectively retards phosphorylated proteins. For assessing the effect of PP2CA on T-loop phosphorylation, immunoprecipitated SnRK1.1-HA (5 µl of beads) was treated or not with GST-PP2CA (1 µg) in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM EDTA, and 1/1000 protease inhibitor cocktail (Sigma P9599) at 30°C for 30 min. The beads were then washed twice with the same buffer complemented with 150 mM NaCl and 0.05% Triton X-100. Finally, they were boiled in Laemmli solubilization buffer and analyzed by immunoblotting with anti–phospho-AMPKα (T172) and anti-SnRK1.1 antibodies.

For in vitro kinase assays, immunoprecipitated SnRK1.1 was pre- incubated (for 10 min) or not with His-PP2CA (0.6 mg) and His-PYL4 (2.0 mg) in 30 µl of kinase buffer (20 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, and 2 mM $MnCl_2$) and ABA (30 µM) and further incubated with GST-DC ABF2 (0.5 mg) for 1h at room temperature in the presence of 3.5 µCi of [γ32P] ATP. The reaction products were resolved in an 8% SDS-PAGE gel, transferred to an Immobilon-P membrane (Millipore), and detected using a phosphor image system (FLA5100; Fujifilm; Antoni et al., 2012).

For preactivation of SnRK1.1, GST-SnRK1.1 and GST-SnAK2 (1 µg of each) were incubated in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 µM ATP, 1 mM DTT, and 1/1000 protease inhibitor cocktail (Sigma) at 30°C for 30 min. After adding or not GST-PP2CA (1 µg), the mix was further incubated for 30 min and analyzed by immunoblot employing anti-phospho-AMPKα (T172) and anti-SnRK1.1 antibodies.

For measurements of endogenous SnRK1 activity, SnRK1.1 was immunoprecipitated from leaves of 4-week-old plants of the indicated genotypes. Plant material (1 g) was extracted in 2 volumes of Buffer C (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.05% Triton X-100), and complete protease inhibitor cocktail (one tablet/50 mL, Roche) and 1/ 500 (v/v) phosphatase inhibitor 2 (Sigma) and 1/500 (v/v) phosphatase inhibitor 3 (Sigma). After two successive centrifugations (20,000g, 4°C, 10 min), the supernatant was

recovered, and 1 mg of total protein was incubated with gentle shaking for 3 h at 4°C with 15 µl beads of protein A–antibody complex prepared as follows. For each immuno-precipitation, 15 µl (bed volume) of protein A–agarose (Roche) was equilibrated in PBS (Sigma-Aldrich) and incubated with 1.5 µg of anti- SnRK1.1 antibody in 500 ml of PBS for 1 h at room temperature with gentle shaking. After three washes in buffer C, the beads were used for immunoprecipitation. After the incubation for 3 h at 4°C under shaking, the beads were washed three times with buffer C, and one-third (5 µL) was kept for immunoblot analysis with an anti-SnRK1.1 antibody.

The remaining 10 µl was used to determine the specific activity of SnRK1 on the AMARA peptide as described previously (Crozet et al., 2010). Briefly, the beads were incubated for 1 h at 30°C in a kinase assay buffer (100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 200 µM ATP, 1 mM EDTA, and 1/500 anti-protease and anti-phosphatase cocktails), 90 µM AMARA peptide (AMARAASAAALARRR), and 2 µCi [γ-32P] ATP. 10 µl of the reaction was spotted three times on P81 filter (GE- Whatman), and the filters were subsequently washed three times for 5 min in 1% phosphoric acid. After a quick wash in acetone, radioactivity was measured using a scintillation counter. A positive control with recombinant SnRK1.1 and SnAK2 was always performed to confirm that the reaction was occurring.

Y2H Assays

Y2H assays were performed as described (Saez et al., 2008). The full-length codingsequenceof SnRK1.1 andthevariousdeletions, clonedintopGBKT7, were faced with constructs harboring full-length PP2CA and ABI1 in fusion with the GAL4 activation domain. To generate the GAL4 activation domain- PP2CA fusion, the PP2CA coding sequence was cloned into pGADT7. The pGADT7-ABI1 construct was described previously (Vlad et al., 2010).

Gene Expression Analyses

Fully expanded leaves of 5-week-old plants were used as such or to cut leaf discs (9-mm diameter) and incubated on sterile MilliQ water in Petri dishes. For examining SnRK1 regulation in wild-type and pp2c mutants, leaves were incubated for 3 h in L (control; 100 µE) or D or DG. Unexpected darkness is perceived as stress and activates SnRK1 (Baena-González et al., 2007). For assessing the effect of ABA, leaf discs of wild-type or 35S: PP2CA plants were incubated 6 ABA under light for 5 h. For the effect of ABA on SnRK1 activation by stress and inactivation by sugar, leaf discs of wild-type plants were incubated for 3 h in light (L), in darkness with (DA) or without ABA (D), or for 1h in darkness followed by 2 h in darkness with ABA and 1h in darkness with ABA and Glc (DGA). Glc and ABA were added to a final concentration of 50 µM and 100 µM, respectively.

Following the indicated treatments, total RNA was extracted using TRIzol reagent (Life Technologies), treated with RNase-Free DNase (Promega), and reverse transcribed (1.5 mg) using SuperScript III Reverse Transcriptase (Life Technologies). qRT-PCR analyses were performed using a 7900HT fast real-time PCR System (Applied Biosystems) employing the Eva-Green fluorescent stain (Biotium), and the 2^{-DCT} or comparative CT method (Livak and Schmittgen, 2001). Expression levels were normalized using the CT values obtained for EIF4. Efficient ABA uptake and signaling was confirmed by monitoring the induction of the ABA marker genes RAB18 and RD29.

Microarray Dataset Comparisons

The dataset for the SnRK1.1-inducedtranscriptional profile corresponds to supplemental table 3 in Baena-González et al. (2007). The hormone treatment data sets, as compared in (Nemhauser et al., 2006), are from the Arabidopsis AtGenExpress consortium (http://Arabidopsis.org/portals/ expression/microarray/ATGenExpress.jsp). A two fold change filter was applied to all the hormone data sets and, given the 6-h incubation of the

SnRK1.1 overexpression data set, only the 3-h (and not the 1-h) time points were considered for the comparisons. Overlap between the compared data sets was revealed using the Venny diagram online application (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The dataset for the SnRK1.1- induced transcriptional profile corresponds to supplemental table 3 in Baena-González et al. (2007). For determining the significance of overlap between the two experiments, hypergeometric testing was applied using the dhyper function in R (http://www.r-project.org/).

Statistical Analyses

All statistical analyses were performed with the GraphPad Prism software. For analyses of qPCR data, the statistical significance of the indicated changes was assessed employing log2-transformed relative expression values (Rieu and Powers, 2009).

Accession numbers

Sequence data from this work can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: SnRK1.1, At3g01090; SnRK1.2, At3g29160; ABI1, At4g26080; PP2CA, At3g11410; ABI2, At5g57050; HAB1, At1g72770; HAI1, At5g59220; PYL4, At2g38310; PP2C6-6, At1g03590; DIN6, At3g47340; SEN5, At3g15450; and AXP, At2g33830.

Chapter III - *A dual function of SnRK2 kinases in plant growth regulation*

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A dual function of SnRK2 kinases in plant growth regulation

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Main text

Adverse environmental conditions trigger responses in plants that promote stress tolerance and survival at the expense of growth. However, little is known of how stress signaling pathways interact with each other and with growth regulatory components to balance growth and stress responses. Here, we show that plant growth is largely regulated by the interplay between the evolutionarily conserved energy-sensing AMPK/SnRK1 protein kinase and the ABA (abscisic acid) phytohormone pathway. While SnRK2 kinases are major drivers of ABA-triggered stress responses, we uncover an unexpected growth-promoting function of these kinases in the absence of ABA as repressors of SnRK1. Sequestration of SnRK1 by SnRK2-containing complexes allows SnRK1 to be released in response to ABA and to mediate TOR inhibition and growth repression by this hormone. This mode of regulation couples growth control with environmental factors typical for the terrestrial habitat and is likely to have been critical for the water-to-land transition of plants.

To cope with adverse environmental conditions, plants trigger cellular and whole-plant responses that confer protection but are often detrimental to growth[1]. Despite the negative impact of stress on crop productivity, how growth is modified by stress signalling pathways is poorly understood. One major component of the stress response is SNF1-related protein kinase 1 (SnRK1), the plant ortholog of yeast SNF1 (Sucrose non-fermenting 1) and mammalian AMPK (AMP-activated protein Kinase), which drives vast metabolic and transcriptional readjustments that restore homeostasis and promote survival[2-4]. Similarly to SNF1 and AMPK, SnRK1 signaling is activated when energy levels decline during stress[2], but is also induced by abscisic acid (ABA)[5], a phytohormone essential for responses to stresses like drought, extreme temperatures or salinity[6]. In the absence of ABA, type 2C phosphatases (PP2Cs) repress subgroup III SnRK2 kinases (SnRK2.2, SnRK2.3, and SnRK2.6 in Arabidopsis thaliana), keeping the pathway inactive[7-11]. Binding of ABA to its receptors enables PP2C sequestration and the release and activation of SnRK2s, which thereby induce protective responses and inhibit growth[12,13].

Numerous studies have suggested cooperation between SnRK1 and ABA signaling in plant stress responses, growth and development[5,14-22], but little is known of the underlying mechanisms. SnRK1 is a heterotrimeric complex and in Arabidopsis the α-catalytic subunit is encoded by two genes, SnRK1α1 and SnRK1α2. To investigate the molecular connection between SnRK1 and ABA signaling and, given the lethality of the double snrk1α1 snrk1α2 knockout[2,23], we generated partial snrk1α1-/- snrk1α2+/- loss-of-function mutants. These mutants show compromised SnRK1 accumulation (Fig. S1) and signaling (Fig. S2), as demonstrated by defective induction of SnRK1 marker genes in response to a transient dark treatment[2]. These are hereafter referred as sesquiα2-1 or sesquiα2-2 mutants, depending on the snrk1α2 allele they harbor.

Despite being mostly similar to the wild-type during early development under normal conditions, sesquiα2 mutants fail to impose an ABA-dependent post-germination growth arrest[24], developing green cotyledons in the presence of the hormone (Fig. 1A, Fig. S3). Furthermore, sesquiα2 mutants are unable to reduce lateral root (LR) number in response to ABA to the same extent as control plants (8-11%, 50%, and 41% of the mock for WT, sesquiα2-1, and sesquiα2-2 seedlings, respectively; Fig. 1B). In similar assays, single snrk1α1 and snrk1α2 mutants are mostly indistinguishable from the wild-type, with only the snrk1α1 mutant being mildly defective in the repression of LR growth in response to ABA (Fig. S4). Other ABA-regulated processes, such as germination (Fig. S5A), primary root (PR) growth (Fig. 1B), transpiration rates (Fig. S5B), and ABA marker gene induction (Fig. S5C) appeared normal in sesquiα2 mutants, suggesting that the lack of SnRK1 affects only specific ABA responses and/or that SnRK1 signaling is not sufficiently compromised to visibly affect all ABA-related processes.

Given that all the observed ABA phenotypes of the SnRK1 sesquiα2 mutants relate to growth repression, and given the known antagonistic relationship between AMPK/SnRK1 and the growth-promoting Target of Rapamycin (TOR) kinase in animals[25] and possibly in plants[4], we examined the activation status of TOR in the sesquiα2-1 mutant in response to ABA. The phosphorylation of ribosomal protein S6 (RPS6S240) in whole seedling

extracts served as a faithful readout[26], confirming previous results on the inhibition of TOR signaling by ABA and its dependency on SnRK2 kinases[27] (Fig. S6). In response to ABA, the sesquiα2-1 mutant showed a slower inhibition of TOR along all the analyzed 4h time-course sampling points (Fig. 1C), indicating that SnRK1α1 is required for repressing TOR activity in response to ABA. To assess if the SnRK1α effect is direct we next analyzed the physical interaction between SnRK1α1 and TOR by co-immunoprecipitation (co-IP), using a GFP-tagged SnRK1α1 line14, a 35S::GFP control line, and antibodies recognizing TOR or its regulatory protein RAPTOR. In whole seedling extracts TOR was readily co-immunoprecipitated with SnRK1α1-GFP (Fig. 1D) but not with GFP alone (Fig. 1E). A basal SnRK1α1-TOR interaction was detected in mock conditions, and it was enhanced two-fold by a short ABA treatment (40 min; Fig. 1D). Similar results were obtained for RAPTOR (Fig. S7A-B), confirming previous observations that SnRK1α1 and RAPTOR interact in planta[4,28]. These results were further corroborated for the endogenous proteins using TOR immunoprecipitation and immunodetection of SnRK1α1 (Fig. S7D). A recent study demonstrated that the repression of TOR by ABA is SnRK2-dependent[27]. However, using a GFP-tagged SnRK2.2 line[29] we were unable to detect any interaction of TOR or RAPTOR with SnRK2.2-GFP either in mock- or ABA-treated plants (Fig. 1F and Fig. S7C). Furthermore, none of the three SnRK2s (SnRK2.2/2.3/2.6) could be detected in immunoprecipitates of endogenous TOR in neither of the two conditions, altogether suggesting that, despite being necessary for repressing TOR in response to ABA[27], SnRK2s may not be directly involved in TOR repression and that TOR is instead inhibited by SnRK1.

Figure 1

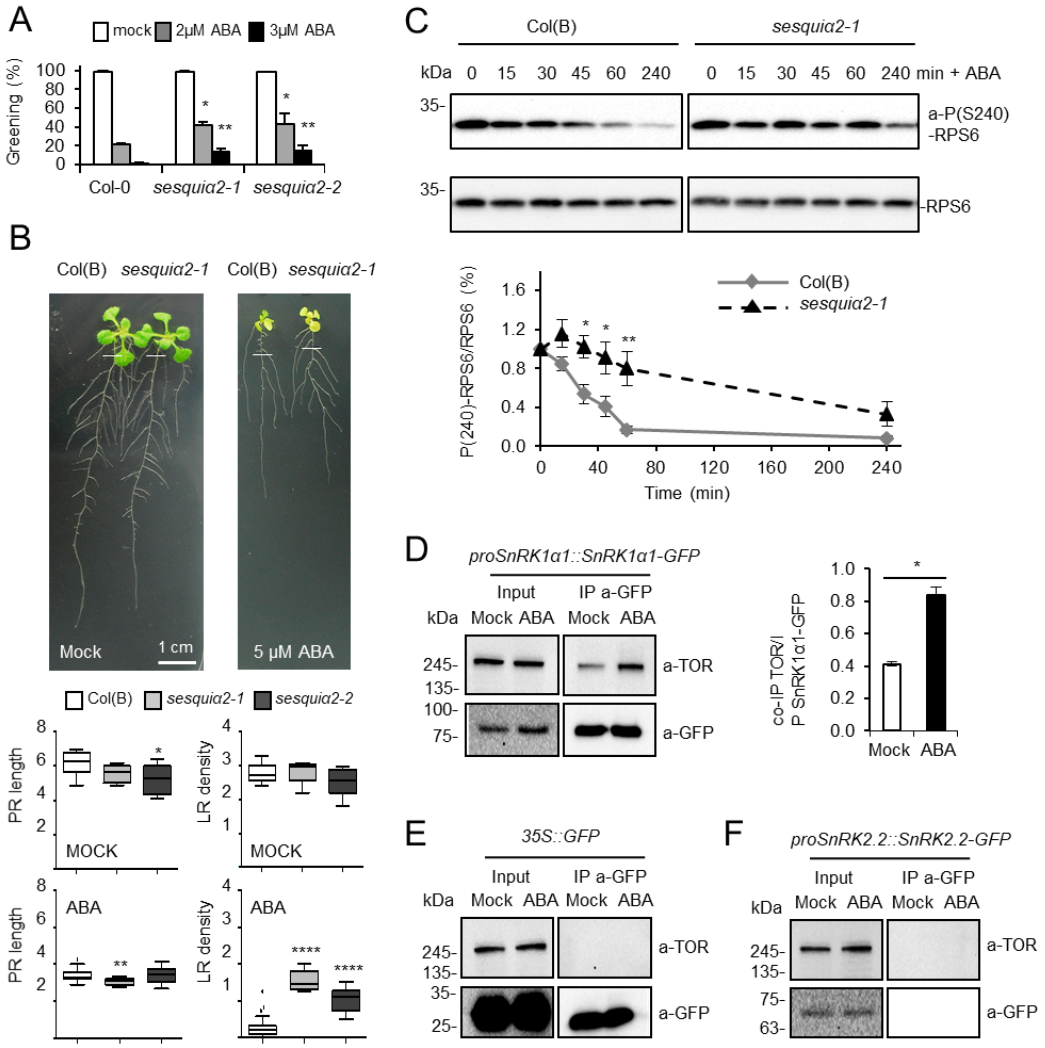

Figure 1. SnRK1 *sesquia2* mutants show defective growth repression in ABA. (A) SnRK1 *sesquia2-1* and *sesquia2-2* mutants have higher cotyledon greening rates than control plants in ABA. Graph shows the percentage of green and expanded cotyledons in seedlings grown for 15d on 0.5X MS with or without ABA (n = 3, 100 seeds per genotype each experiment; error bars, SEM). Statistically significant differences relate to comparisons to control plants (one-way ANOVA with Tukey HSD test). **(B)** SnRK1 *sesquia2-1* and *sesquia2-2* mutants have higher lateral root (LR) density than control plants in ABA. Upper panels, representative pictures of seedlings grown

vertically on 0.5X MS medium with BASTA for 5d and transferred to 0.5X MS with or without ABA for 8d. Lower panels, quantification of primary root (PR) length and LR density from 4 independent experiments (total number of plates: WT mock n=16, *sesquiα2-1* mock n=7, *sesquiα2-2* mock n=9, WT ABA n=24, *sesquiα2-1* ABA n=12, *sesquiα2-2* mock n=12; total number of seedlings: 36-72 per genotype and condition). Col(B), BASTA-resistant Col-0 control plants (*35S::GFP*). Statistically significant differences relate to comparisons to control plants (one-way ANOVA with Tukey HSD test). **(C)** Repression of TOR signaling in response to ABA is slower in SnRK1 *sesquiα2-1* mutants than in Col(B) control plants. Seedlings were treated with 50 µM ABA for the indicated times and TOR activity was subsequently analyzed from total protein extracts using immunoblotting and $RPS6^{S240}$ phosphorylation as readout. Graph corresponds to the average of 5 independent experiments (error bars, SEM; two-tailed Student t-test); **(D)** TOR interacts with SnRK1α1 and the interaction is enhanced two-fold in ABA. 14d-old seedlings expressing SnRK1α1-GFP, were treated with mock or 50 µM ABA for 40 min, GFP-tagged proteins were immunoprecipitated from total protein extracts and co-immunoprecipitation of TOR was assessed by immunodetection with TOR specific antibodies. Graph corresponds to the average of 2 independent experiments (error bars, SEM; two-tailed Student t-test). TOR is not co-immunoprecipitated with GFP alone **(E)** or with SnRK2.2-GFP **(F)**. 14d-old seedlings expressing *35S::GFP* or *proSnRK2.2::SnRK2.2-GFP* were treated and analyzed as in **(D).** (*), p-value<0.05; (**), p-value<0.01.

To explore the molecular connection between SnRK2 and SnRK1, we first examined their potential co-localization. As previously reported, SnRK1α1 and SnRK2.2 were prominently expressed in the root tip, in LR primordia and in subsequent stages of LR development (Fig. 2A)[14,29]. At the subcellular level both kinases were present in the cytosol and the nucleus, being particularly enriched in the latter (Fig. 2A). To investigate the SnRK1-SnRK2 physical interaction we next performed reciprocal co-IP experiments using the same material and conditions as for the microscopy analyses (roots, 3h ABA treatment). In mock-treated seedlings we retrieved a clear interaction between SnRK1α1 and SnRK2 in both directions (Fig. 2B-2C), whilst neither SnRK2 nor SnRK1α1 could be detected in immunoprecipitates of GFP alone (Fig. S8A). The reported interaction of both SnRK2[9,10] and SnRK1α1[5] with clade A PP2C phosphatases served as positive controls (Fig. S9). Strikingly, treatment with ABA caused a marked reduction in all three interactions (SnRK2-SnRK1α1, Fig. 2B-2C; PP2CA-SnRK2 and PP2CA-SnRK1α1, Fig. S9; note that this is relative to the total PP2CA amount, which is known to be readily increased by ABA through transcriptional activation[30]), suggesting that the three proteins may be part of the same complexes. A similar effect of ABA on the SnRK2-SnRK1α1 interaction was observed using the same material and conditions as for evaluating the interaction with TOR (whole seedlings, 40 min ABA treatment; Fig. S8B-C), showing the interaction is readily reduced by the hormone.

Figure 2

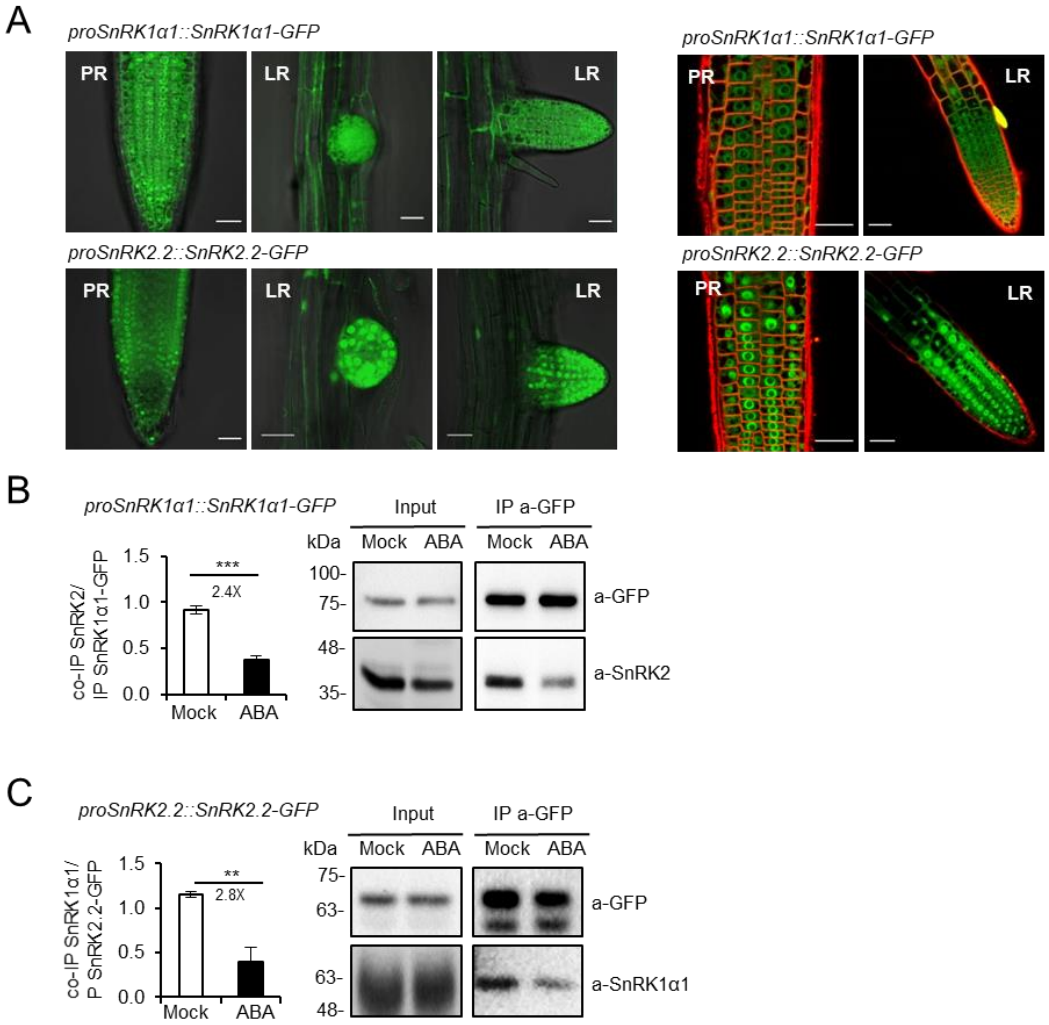

Figure 2. SnRK2s interact physically with SnRK1. (A) Left panels, SnRK1α1 and SnRK2.2 are expressed in the primary root (PR) and during lateral root (LR) development. Right panels, SnRK1α1 and SnRK2.2 are highly enriched in the nucleus. Roots were stained with FM4-64. **(B, C)** SnRK1α1 and SnRK2.2 interact *in planta* and the interaction is reduced over 2-fold in ABA. Seedlings expressing *proSnRK1α1:SnRK1α1-GFP* **(B)** or *proSnRK2.2:SnRK2.2-GFP* **(C)** were mock- or ABA-treated, GFP-tagged proteins were immunoprecipitated from roots and co-immunoprecipitation of SnRK2 and SnRK1α1, respectively was assessed by immunodetection with the indicated antibodies. Graphs correspond to the average of 4 independent experiments (error bars, SEM; two-tailed Student t-test). (**), p-value<0.01; (***), p-value<0.001.

To investigate the relationship between SnRK1 and SnRK2 kinases we crossed the snrk1α1 single mutant to the snrk2.2/2.3 double mutant (hereafter referred as snrk2d) to assess their genetic interaction (Fig. S10). We reasoned that, given the partial impairment of ABA responses in this mutant7 [as opposed to the full impairment of the snrk2.2/2.3/2.6 mutant (snrk2t)[31-33]], a potential contribution from the snrk1α1 mutation could be more easily detected in this background. Despite having mostly no effect on its own (Fig. S4), the snrk1α1 mutation clearly enhanced the ABA insensitivity of the snrk2d mutant, increasing its germination and cotyledon greening rates (Fig. 3A), and the formation of LRs in ABA (Fig. 3B). This indicates that the SnRK1 pathway contributes to specific ABA signaling outputs.

We next asked whether repression of TOR by SnRK1 always requires SnRK2s or whether this requirement is specific to ABA. To address this we compared the inhibition of TOR by a dark-induced energy deficit in control plants, sesquiα2-1, and snrk2t mutants. As expected, sesquiα2-1 seedlings had a reduced capacity to repress RPS6S240 phosphorylation in response to darkness (Fig. S11A). This is consistent with previous reports showing defective repression of TOR outputs in plants that have compromised SnRK1 signaling[4]. However, the snrk2t mutant displayed similar kinetics in the repression of TOR signaling as the wild-type (Fig. S11B), supporting the idea that SnRK2s are only required for repressing TOR via SnRK1 in response to ABA but not energy depletion.

We noticed that, despite its ABA insensitivity and overall increased growth in ABA, the snrk2d mutant displayed reduced PR and LR growth in control plates compared to the WT (Fig. 3B), in accordance with a previous report[29]. Most strikingly, this was fully rescued by the snrk1α1 mutation, indicating that the reduced growth of the snrk2d mutant is SnRK1α1-dependent and suggesting that, in the absence of ABA, SnRK2s promote root growth by repressing SnRK1α1 (Fig. 3B). Further supporting a growth-promoting function of SnRK2s in ormal conditions, a line overexpressing SnRK2.3 had longer PR in control plates (Fig. S12), whilst showing enhanced repression of PR growth in ABA, in accordance with its known ABA hypersensitivity[34].

Figure 3

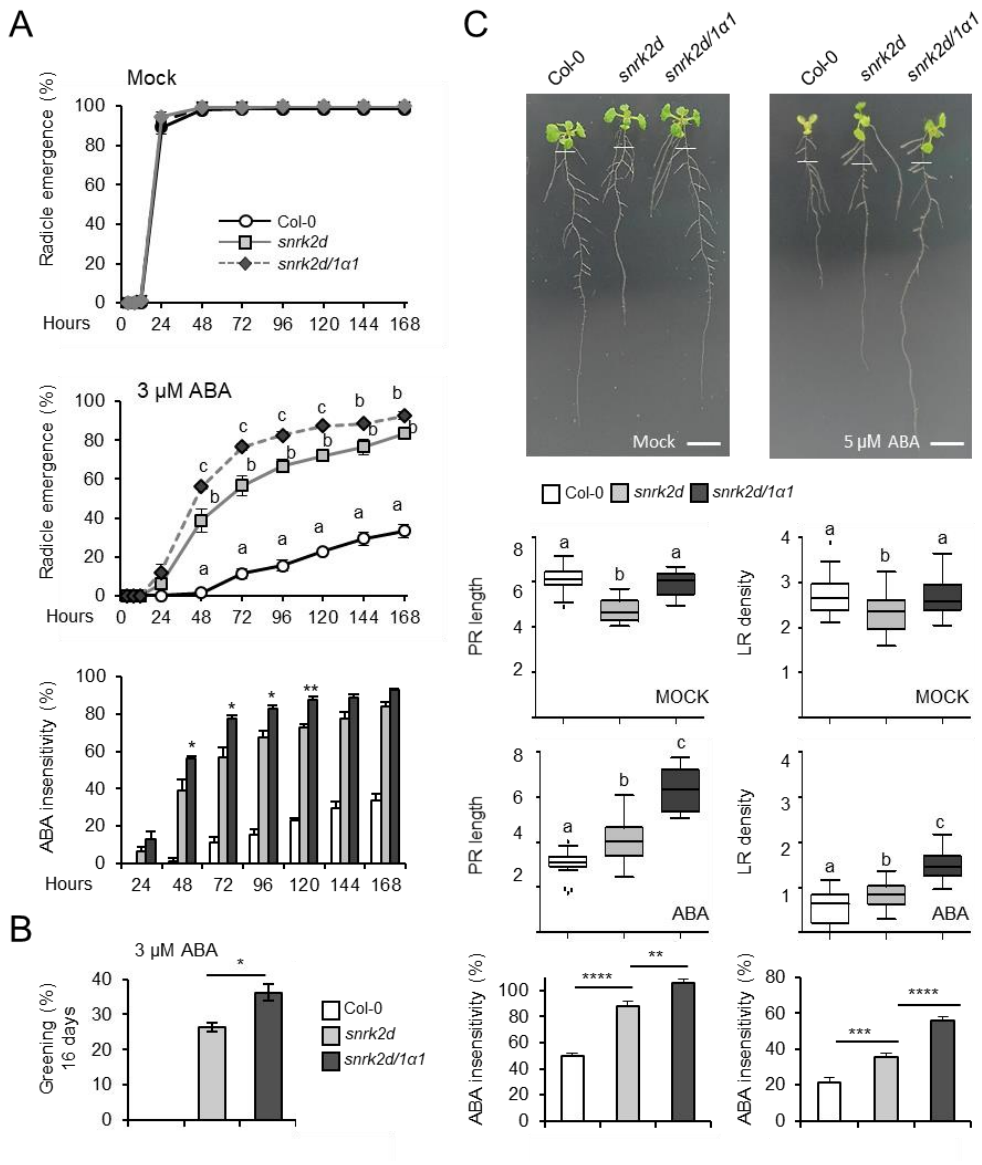

Figure 3. SnRK2s regulate growth *via* SnRK1. (A) The *snrk1α1-3* mutation increases the ABA insensitivity of the *snrk2d* mutant during germination. Seeds of Col-0, *snrk2d*, and *snrk2d snrk1α1* (*snrk2d/1α1*) mutants were plated on 0.5X MS with or without ABA and radicle emergence was scored at the indicated times (shown are percentages in ABA as compared to the mock condition; n=3, 50 seeds per genotype each; error bars, SEM). Different letters indicate statistically significant differences for each time point ($p<0.05$, one-way ANOVA with Tukey HSD test). Lower panel, degree of ABA insensitivity computed by normalizing the parameters scored in ABA to the corresponding mock control (error bars, SEM; p-values refer to the differences between *snrk2d/1α1* and *snrk2d*, one-way ANOVA with Tukey HSD test for each time point). **(B)** The *snrk1α1-3*

mutation increases the cotyledon greening rates of the *snrk2d* mutant in ABA. Seeds were grown as in **(A)** and cotyledon greening was scored after 16d. (*), p< 0.05, Student t-test. **(C)** In control conditions the *snrk2d* mutant has defects in primary (PR) and lateral root (LR) growth that are fully rescued by the *snrk1α1* mutation. In ABA the *snrk1α1* mutation enhances the ABA hyposensitivity of the *snrk2d* mutant with regard to PR length and LR density. Upper panel, representative picture of seedlings grown vertically on 0.5X MS medium for 5d and transferred to 0.5X MS with or without ABA for 8d. Bar = 1cm. Middle panels, quantification of PR length and LR density from 3 independent experiments (total number of plates: WT mock n=21, *snrk2d* mock n=19, *snrk2d/1α1* mock n=21, WT ABA n=21, *snrk2d* ABA n=21, *snrk2d/1α1* ABA n=21; total number of seedlings: 42-44 seedlings per genotype and condition). Different letters indicate statistically significant differences (p<0.05, one-way ANOVA with Tukey HSD test). Lower panels, degree of ABA insensitivity computed by normalizing the parameters scored in ABA to the corresponding mock control (error bars, SEM; p-values refer to the differences between *snrk2d/1α1* and *snrk2d*, one-way ANOVA with Tukey HSD test for each time point). (*), p< 0.05; (**), p<0.01; (***), p<0.001.

We conclude that SnRK2 kinases perform dual functions in plants (Fig. 4). In the absence of ABA, SnRK2s are required to form repressor complexes that block SnRK1 activity and thereby promote growth. Sequestration of SnRK1α1 in these complexes is important for root growth (in the case of SnRK2.2 and SnRK2.3), and may potentially explain other reported unexpected effects of SnRK2 kinases, including SnRK2.6, in promoting metabolism, growth, and development in optimal conditions[35,36]. We propose that these complexes are the same as the ones performing canonical ABA signaling functions and that their disassembly requires sequestration of the PP2C repressors by the ABA-bound ABA receptors. First, likewise SnRK2s[37], the activation of SnRK1 by ABA requires relief of inhibition by PP2C phosphatases[5]. Second, ABA reduced the interaction of SnRK1α1 with SnRK2 and PP2CA (Figs 2B-C, S8B-C, S9A) and between SnRK2 and PP2CA (Fig. S9B). Third, SnRK2s (SnRK2.2/SnRK2.3/SnRK2.6) are absolutely required for repressing TOR in response to ABA[27] (Fig. S6B), even though SnRK2s may be involved in TOR repression only indirectly.

In the presence of ABA these repressor complexes dissociate, releasing SnRK1α1 and SnRK2 to activate stress responses. One major consequence of the ABA-triggered disassembly of these complexes is the increased interaction of SnRK1α1 with TOR to inhibit growth. In agreement with this, Arabidopsis raptor and lst8 mutants are ABA hypersensitive with regard to germination, early seedling development, and root growth[38,39] whilst TOR overexpressors in rice display ABA insensitivity during germination[40]. The fact that the ABA sensitivity of the sesquiα2 mutants was only manifested at the level of cotyledon greening and LR density but not at the level of germination or PR length (Fig. 1), is likely to be explained by the weak nature of these mutants (Fig. S2), by the fact that germination had to be scored from a segregating seed population and by the fact that LRs are more sensitive to ABA than the PR[41]. Repression of TOR in response to ABA may also require active input from SnRK2[27]. However, given the lack of interaction between SnRK2s and TOR in planta (Fig. 1F and Fig. S7), the simple requirement of SnRK2s to form SnRK1 repressor complexes that disassemble in response

to ABA may be sufficient to explain why SnRK2s are essential for growth repression by this hormone[27].

Repression of SnRK1 by SnRK2 and PP2C allows SnRK1 to be released and activated in response to ABA. However, SnRK1 is also regulated by energy signals through mechanisms that are SnRK2-independent (Fig. S11), suggesting that SnRK1 associates with different factors that enable its activation in response to specific signals. We propose that, in addition to its ancient and highly conserved energy-sensing function, SnRK1 evolved in land plants to respond to ABA, a crucial signal for survival in terrestrial habitats. Intriguingly, this is accomplished through repression by the phylogenetically related subgroup III SnRK2 kinases, which belong to the same SnRK superfamily as SnRK1[42], but are specific to land plants[43,44]. Coupling the ABA-PP2C-SnRK2 module to the evolutionarily conserved SnRK1-TOR axis conferred plants the capacity to regulate growth in response to water availability and may have represented a stepping stone for the establishment of terrestrial life.

Acknowledgements

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Figure 4

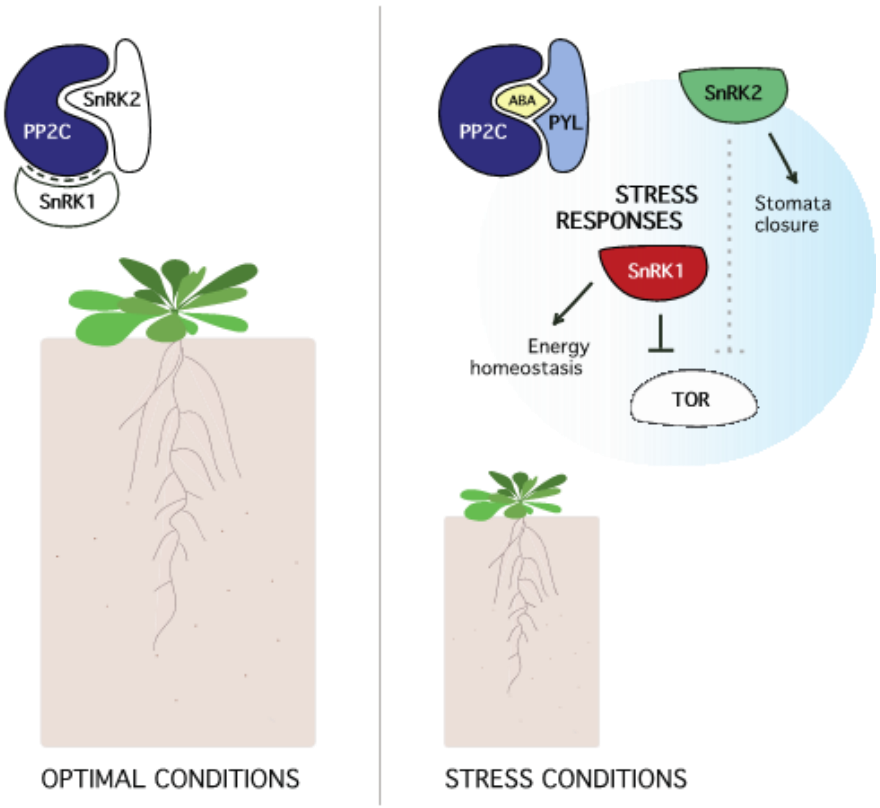

Figure 4. A dual function of SnRK2 kinases in the regulation of growth. (A) In the absence of ABA SnRK2s are required for the formation of specific SnRK1 repressor complexes. Sequestration of SnRK1 in these complexes is important to allow growth under optimal conditions. **(B)** In the presence of ABA these repressor complexes disassemble through canonical ABA signaling, releasing SnRK2s and active SnRK1α to activate stress responses and inhibit growth. This is partly accomplished by direct TOR repression by SnRK1 but may also involve active co-participation of SnRK2 kinases.

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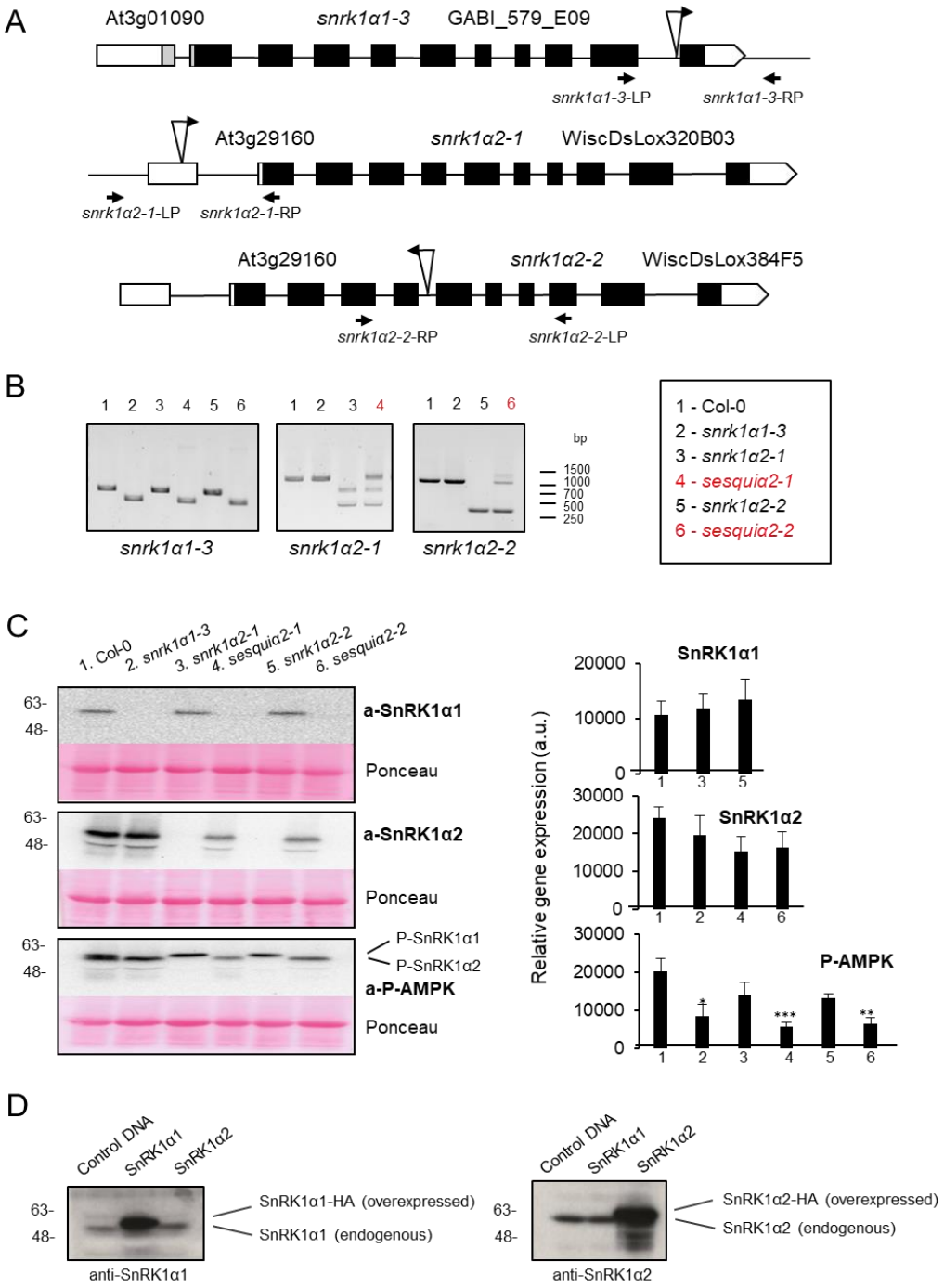
Supplementary material

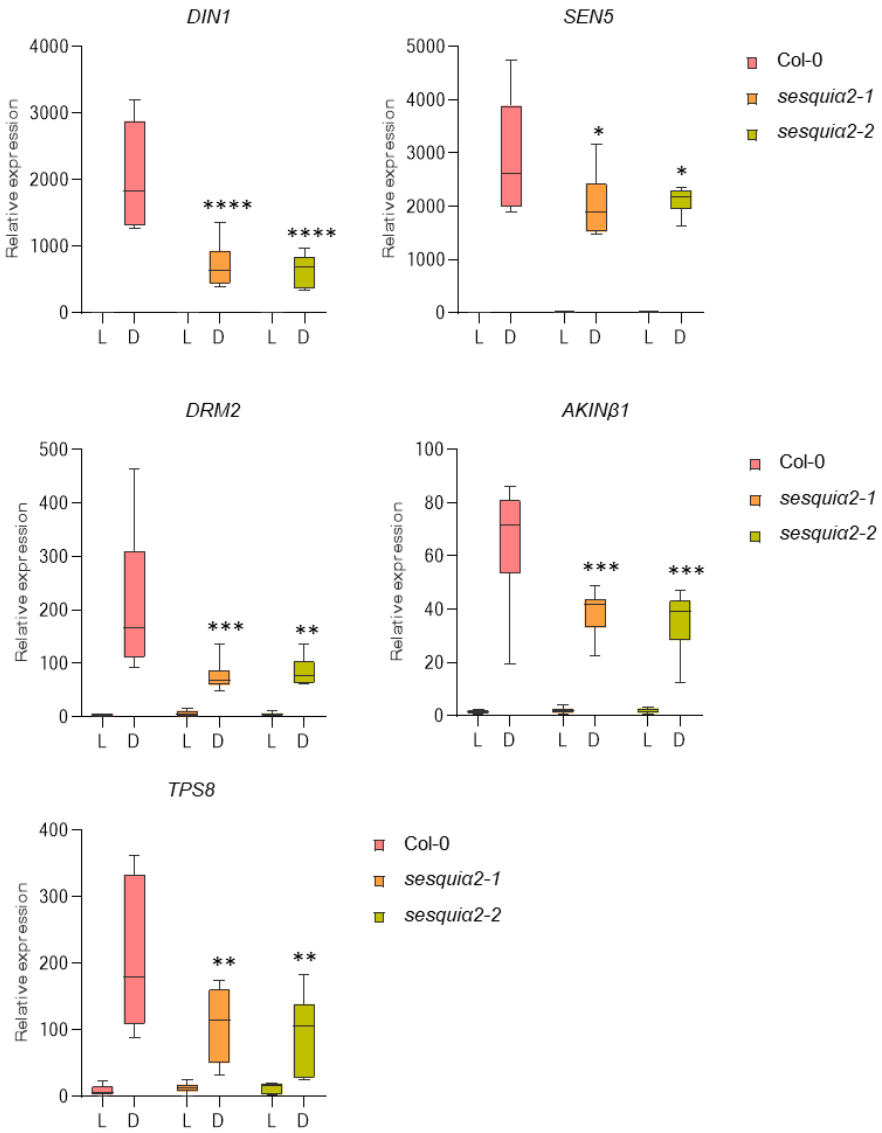
Supplementary Figure 1



Supplementary Figure 1. Generation of SnRK1 *sesquiα2* mutants. (A) Scheme showing the insertion sites of the *snrk1α1 and snrk1α2* T-DNA mutants used in this study. **(B)** Confirmation of *sesquiα2* mutant identity by genotyping. Lanes containing samples from *sesquiα2-1* and *sesquiα2-2* mutants (lanes 4 and 6, respectively) are markerd in red. **(C)** Accumulation of SnRK1α1 and

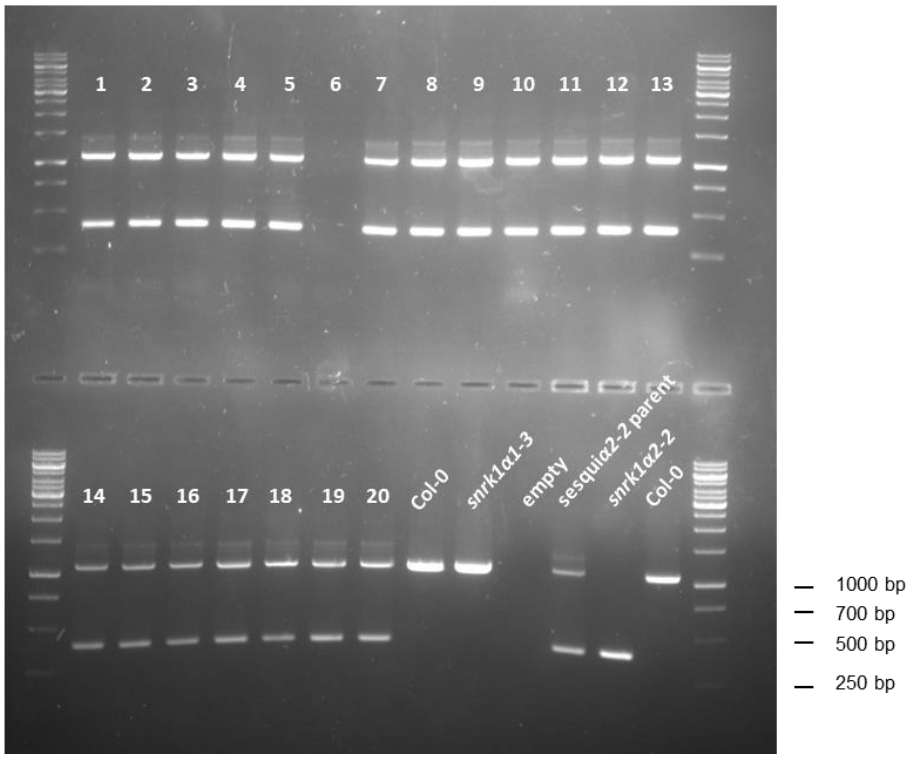
SnRK1α2 proteins is defective in the *sesquiα2* mutants. Left panels, representative blots showing the accumulation of SnRK1α1 and SnRK1α2 in the indicated genotypes. SnRK1α T-loop phosphorylation is detected with a phospho-AMPK antibody (P-AMPK). Ponceau staining of membranes shows equal protein loading in all samples. Right panel, quantification of indicated proteins from 2 independent experiments (each with 3 technical replicates; error bars, SEM). Numbers refer to the genotypes shown on the right panel. Asterisks denote statistically significant differences (one-way ANOVA with Tukey HSD test). (*), $p < 0.05$; (**), $p > 0.01$, (***), $p < 0.001$. **(D)** Specificity of the SnRK1α antibodies described in this study. Anti-SnRK1α1 recognizes the SnRK1α1 but not the SnRK1α2 protein overexpressed in Arabidopsis protoplasts. Conversely, anti-SnRK1α2 recognizes the SnRK1α2 but not the SnRK1α1 overexpressed protein.

Supplementary Figure 2



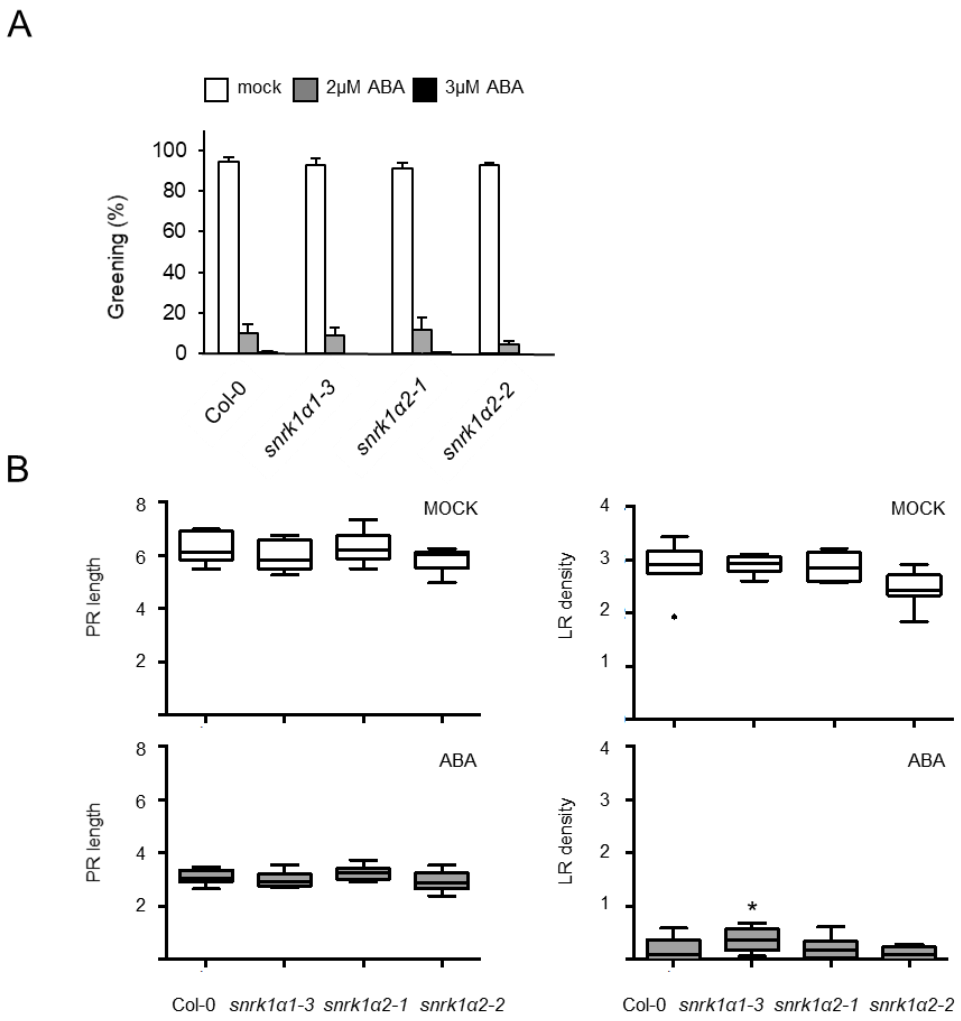
Supplementary Figure 2. SnRK1 *sesquiα2* mutants show defective SnRK1 signaling. BASTA-selected *sesquiα2-1* and *sesquiα2-2* plants were grown on soil for 4 weeks under a 12:12h photoperiod. Rosette leaves were detached and incubated on sterile MilliQ water in covered Petri dishes under light (control; 100 µmol $m^{-2}s^{-1}$) or darkness (energy stress) for 6h (starting 3h after the lights are on). qPCR analyses show defective induction of the indicated SnRK1 marker genes in darkness in the *sesquiα2-1* and *sesquiα2-2* mutants compared to the WT control. Asterisks denote statistically significant differences (two-way ANOVA with Dunnett's multiple comparison test). (*), $p < 0.05$; (**), $p > 0.01$, (***), $p < 0.001$.

Supplementary Figure 3



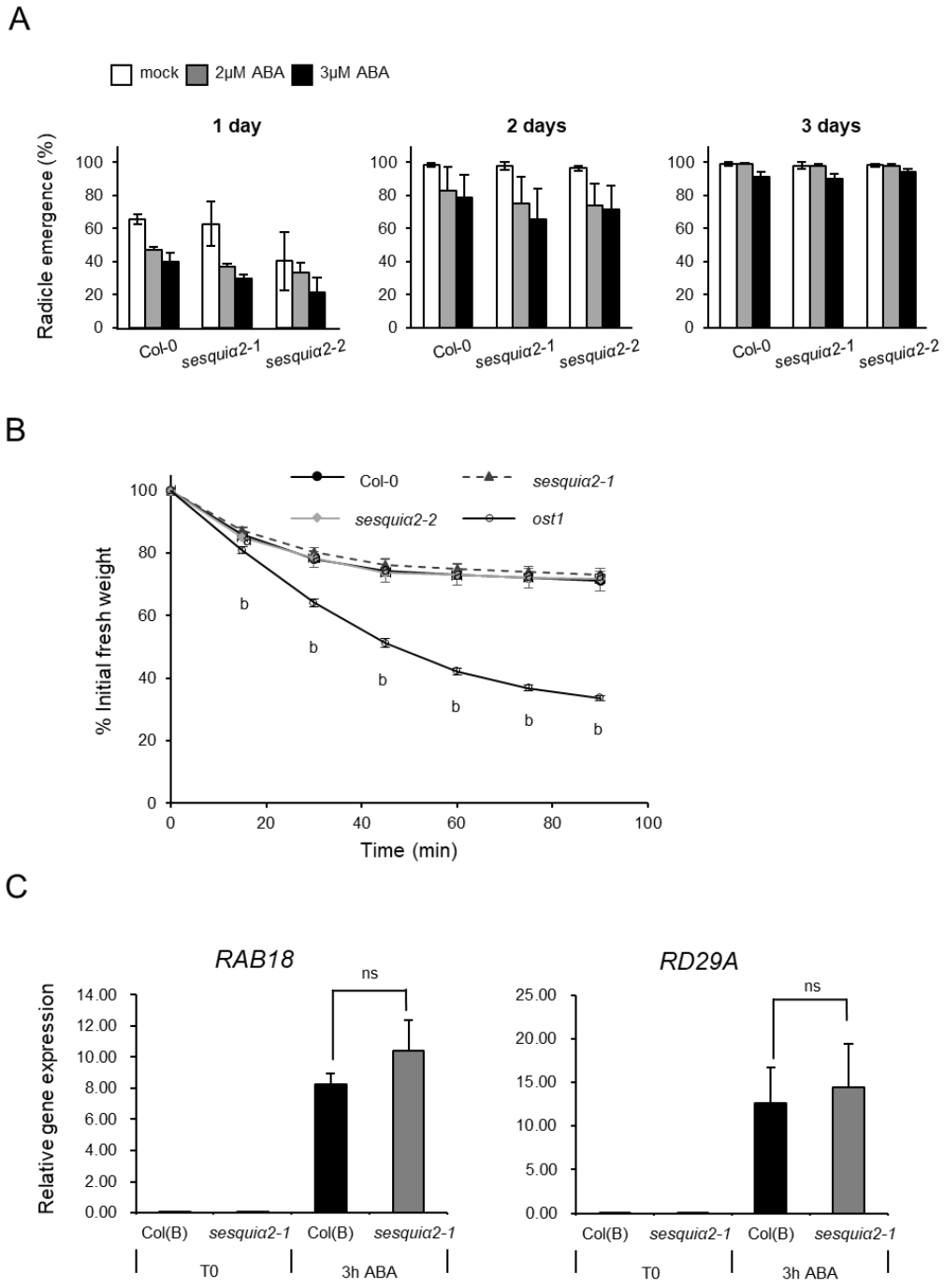
Supplementary Figure 3. Progeny from *sesquiα2* plants that develop green cotyledons in ABA have a *sesquiα2* genotype, as exemplified by analyses of the *sesquiα2-2* mutant. The *sesquiα2-2* mutant has the *snrk1α2-2* mutation in heterozygosity, and hence its seeds are a mixed population of *sesquiα2-2* and single *snrk1α1-3* mutants (50:50, Confraria et al., in preparation). For assays of ABA sensitivity during early seedling development, seeds from s*esquiα2-2* plants cannot be preselected on BASTA to identify true *sesquiα2* seedlings and have to be instead plated directly on medium with or without ABA (2 µM). However, after 15d, only *sesquiα2-2* seedlings develop green cotyledons in ABA, as shown by the genotyping analyses of twenty randomly selected seedlings with green cotyledons. Genotyping PCR was for *snrk1α2-2* (see materials and methods), the allele segregating in the *sesquiα2-2* plants.

Supplementary Figure 4



Supplementary Figure 4. Single *snrk1α1 and snrk1α2* mutants have mostly normal ABA sensitivity. (A) Quantification of green and expanded cotyledons of SnRK1 single mutants (*snrk1α1-3*, *snrk1α2-1, snrk1α2-2*) and Col-0 wild-type seedlings grown on 0.5X MS with or without ABA for 15d. Percentage of green and expanded cotyledons in ABA as compared to the mock condition (average from 3 independent experiments, 100 seeds per genotype each; error bars, SEM). **(B)** Quantification of primary root (PR) length and LR density from at least 2 independent experiments shows only a mild ABA hyposensitivity in the *snrk1α1-3* mutant with regard to LR density (total number of plates: WT mock n=8, *snrk1α1* mock n=8, *snrk1α2-1* mock n=8, *snrk1α2-2* mock n=8, WT ABA n=17, *snrk1α1* ABA n=17, *snrk1α2-1* ABA n=17, *snrk1α2-2* ABA n=10; total number of seedlings: 32-60 seedlings per genotype and condition). (*), $p < 0.05$; one-way ANOVA with Tukey HSD test.

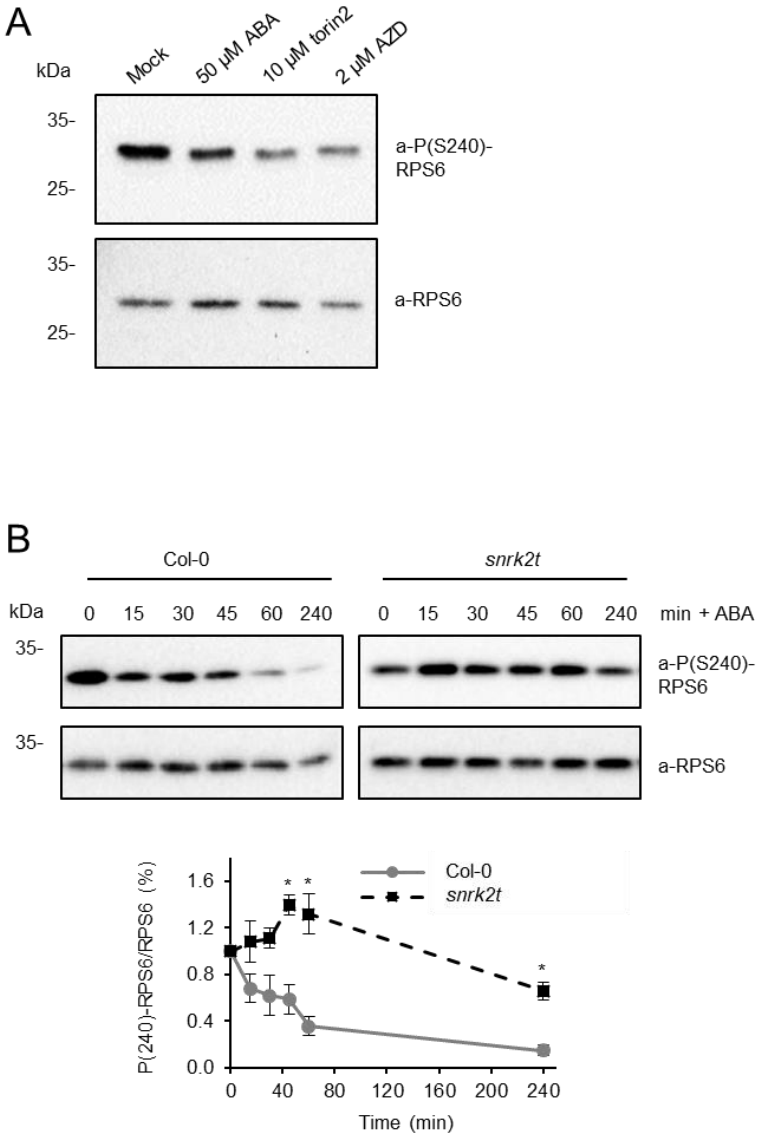
Supplementary Figure 5



Supplementary Figure 5. Several ABA responses are normal in SnRK1 *sesquiα2* mutants. (A) SnRK1 *sesquiα2* mutants show normal ABA sensitivity during germination. Seeds of the indicated genotypes were plated on 0.5X MS with or without ABA and radicle emergence was scored for the

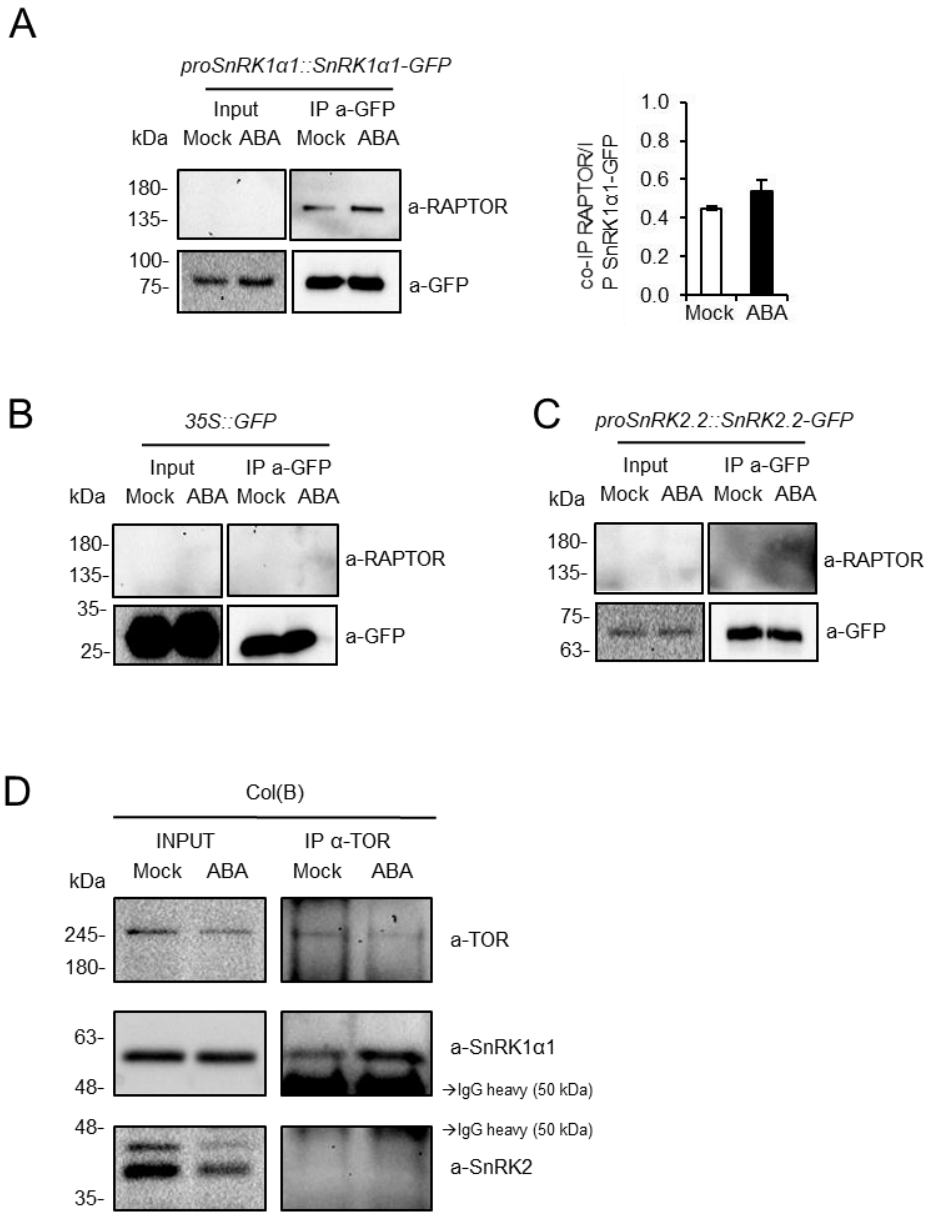
indicated times (percentage in ABA as compared to the mock condition). Shown are average values from 3 independent experiments (each with 100 seeds per genotype; error bars, SEM; one-way ANOVA with Tukey HSD test). **(B)** SnRK1 *sesquiα2* mutants show normal water loss rates. Leaves of similar age were detached from 30d-old plants of the indicated genotypes (5 leaves from 5 independent plants), weighed, subjected to the drying atmosphere of a laminar flow hood, and re-weighed at the indicated times. Values are averages of the percentage of initial fresh weight (error bars, SEM). Different letters indicate statistically significant differences ($p<0.05$, one-way ANOVA with Tukey HSD test). **(C)** SnRK1 *sesquiα2* mutants show normal induction of ABA marker genes. Levels of *RAB18* and *RD29A* were measured by qPCR from 14d-old seedlings growing on 0.5X MS and mock- or ABA-treated (50 µM) for 3h (error bars, SEM; two-tailed Student t-test). (ns), non significant; (*), p-value<0.05; (**), value<0.01.

Supplementary Figure 6



Supplementary Figure 6. Use of RPS6^{S240} phosphorylation to monitor TOR inhibition by ABA. (A) Treatment of 11d-old seedlings with 50 µM ABA, 10 µM torin2 or 2 µM AZD8055 during 3h induces a strong repression of TOR activity as evidenced by the reduced RPS6^{S240} phosphorylation levels. **(B)** Lack of SnRK2.2, SnRK2.3, and SnRK2.6 in the *snrk2t* mutant abrogates the repression of TOR activity by ABA. Col-0 seedlings reach nearly full TOR repression within 4h, whereas no changes in TOR activity can be observed in *snrk2t* seedlings within this timeframe. Graph corresponds to the average of 3 independent experiments (error bars, SEM; two-tailed Student t-test). (*), p-value<0.05.

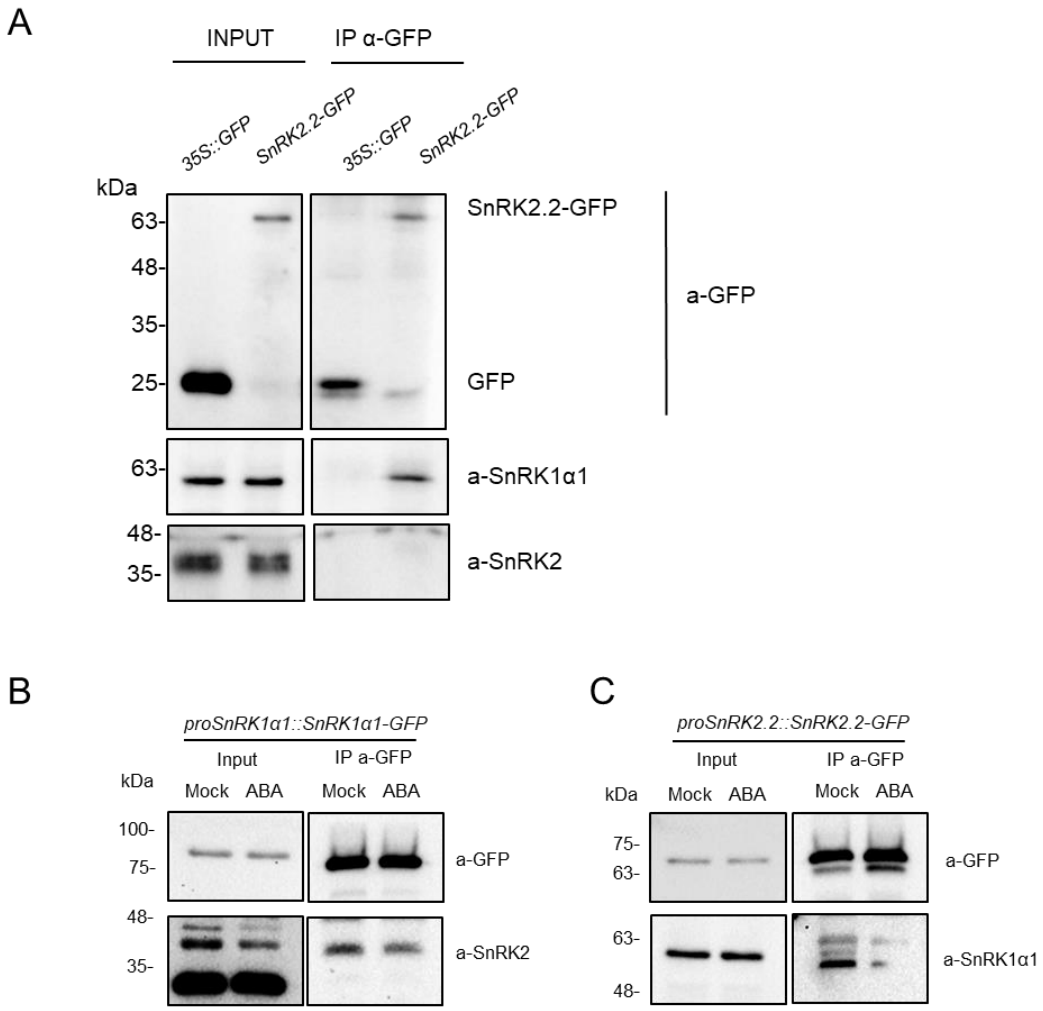
Supplementary Figure 7



Supplementary Figure 7. RAPTOR and TOR interact with SnRK1α1. (A) RAPTOR interacts with SnRK1α1 in mock and ABA. 14d-old seedlings expressing *proSnRK1α1::SnRK1α1-GFP* were treated with mock or 50 µM ABA for 40 min, GFP-tagged proteins were immunoprecipitated from total protein extracts and co-immunoprecipitation of RAPTOR was assessed by immunodetection with RAPTOR-specific antibodies. Graph corresponds to the average of two independent experiments (error bars, SEM). RAPTOR does not co-immunoprecipitate with GFP alone **(B)** or

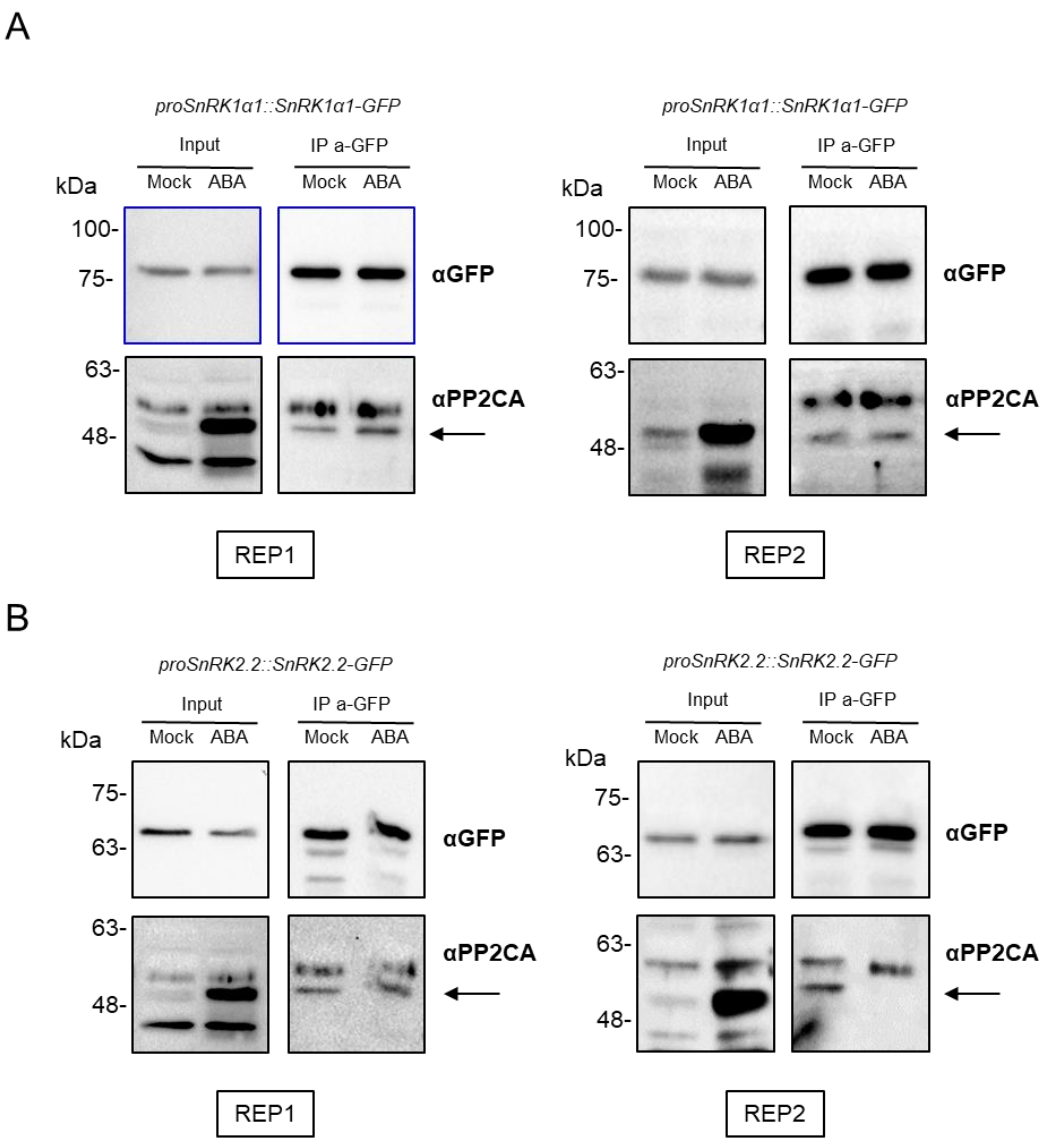
with SnRK2.2-GFP **(C)**. **(D)** Reciprocal immunoprecipitation assays corroborate the SnRK1α1-TOR interaction. 14d-old seedlings were treated with mock or 50 µM ABA for 40 min, endogenous TOR was immunoprecipitated from total protein extracts using TOR specific antibodies and co-immunoprecipitation of SnRK1α1 and SnRK2s was assessed by immunodetection with specific antibodies.

Supplementary Figure 8



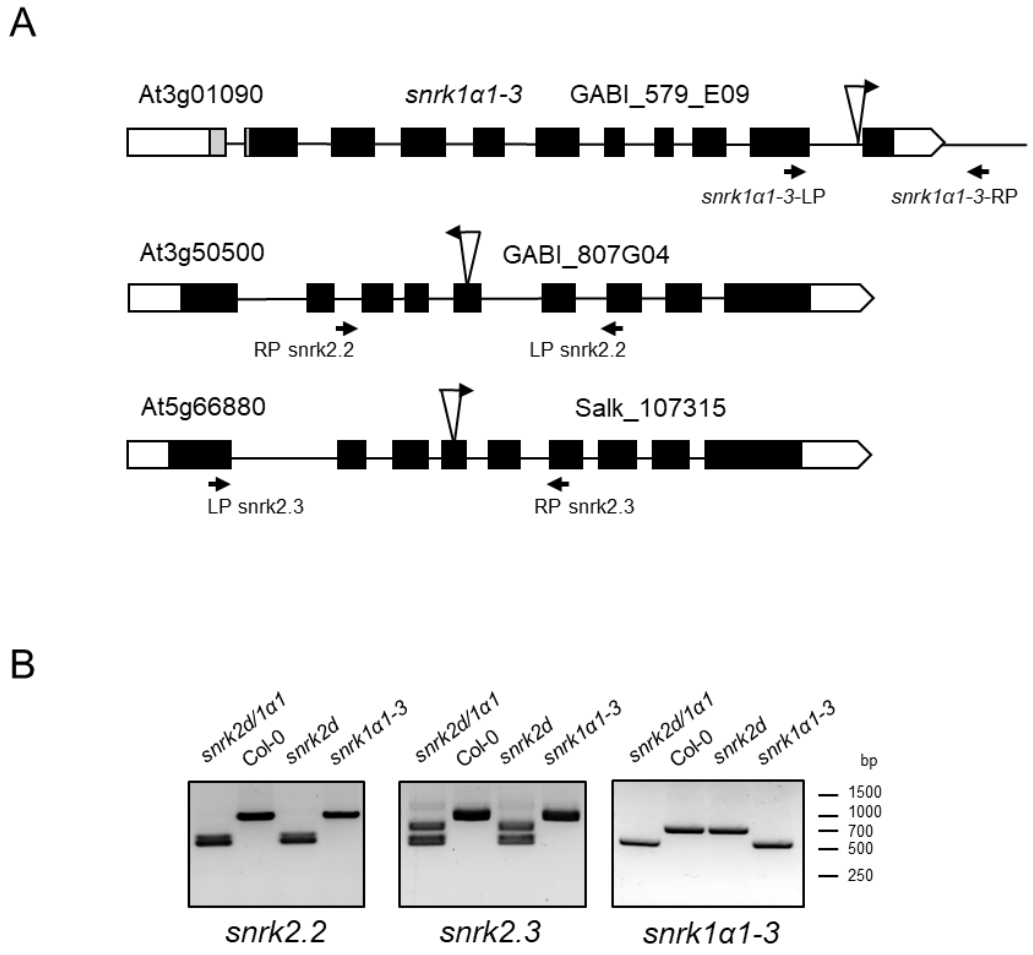
Supplementary Figure 8. SnRK1 and SnRK2 kinases interact *in planta*. (A) SnRK1α1 and SnRK2s do not co-immunoprecipitate with GFP alone in roots of seedlings grown in 0.5X MS. **(B-C)** The interaction between SnRK1α1 and SnRK2 is detected also in extracts from whole seedlings (**B**, IPs from *proSnRK1α1::SnRK1α1-GFP* seedlings; **C**, IPs from *proSnRK2.2::SnRK2.2-GFP* seedlings) and the interaction is reduced upon a short ABA treatment (40 min, 50 µM).

Supplementary Figure 9



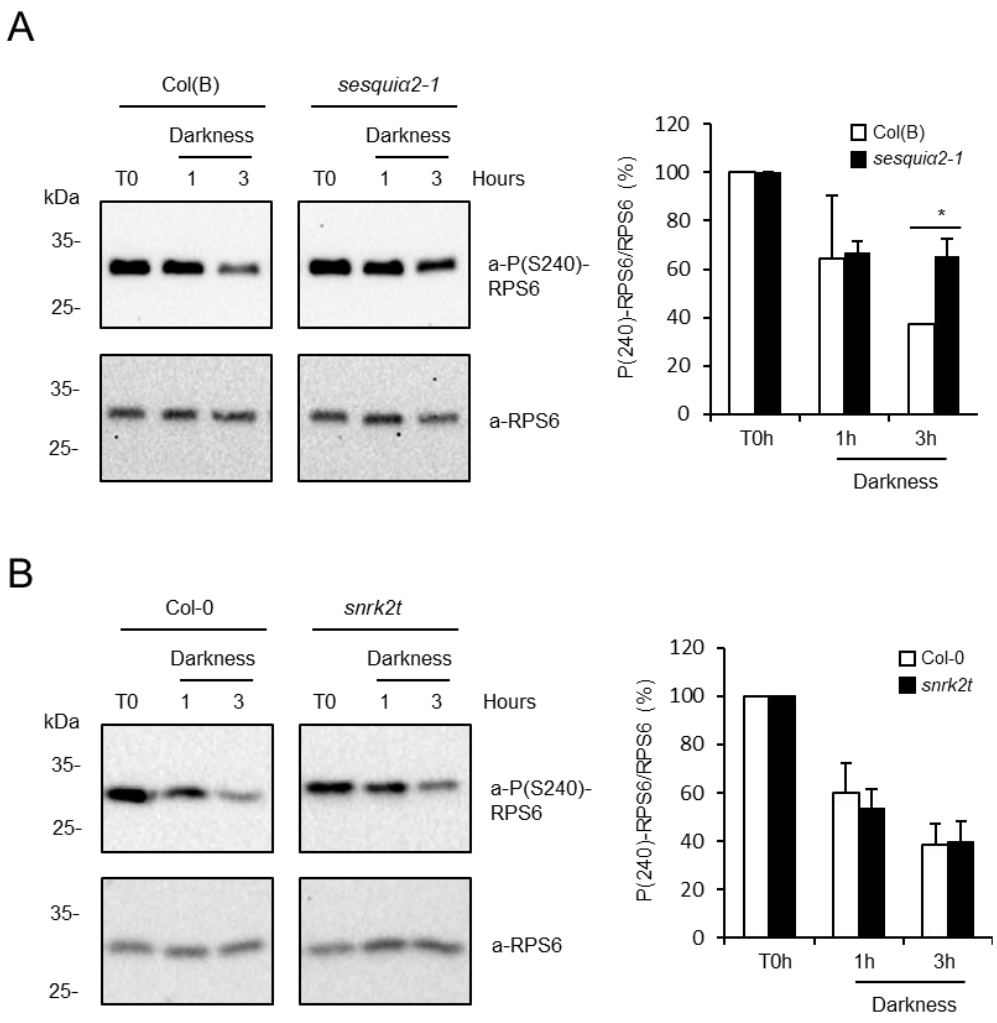
Supplementary Figure 9. PP2CA interacts with SnRK1 and SnRK2 *in planta*. PP2CA co-immunoprecipitates with SnRK1α1-GFP **(A)** and SnRK2.2-GFP **(B)** and, proportionally to the total PP2CA levels, both interactions are reduced in ABA. Seedlings expressing *proSnRK1α1::SnRK1α1-GFP* or *proSnRK2.2::SnRK2.2-GFP* were mock- or ABA-treated, GFP-tagged proteins were immunoprecipitated from roots and co-purifying proteins were analyzed by immunoblotting with specific antibodies. Blots marked in blue correspond to the same samples of Figure 2B.

Supplementary Figure 10



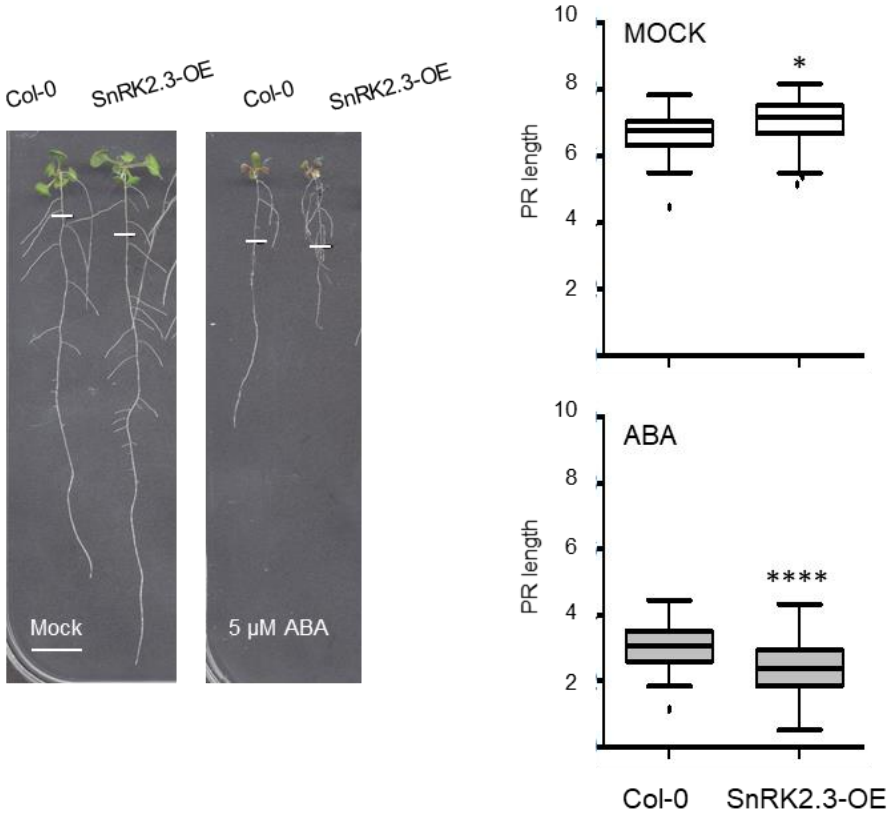
Supplementary Figure 10. Generation of the *snrk2d/1α1* mutant. (A) Scheme showing the insertion sites of the *snrk1α1, snrk2.2* and *snrk2.3* T-DNA mutations of the parental lines. **(B)** Confirmation of the *snrk2d/1α1* mutant identity by genotyping. The *snrk2d/1α1* mutant was generated by crossing the *snrk2d* (*snrk2.2 snrk2.3*) and *snrk1α1-3* mutants. F2 individuals able to grow on 1 µM ABA were genotyped for the *snrk1α1-3* mutation and plants homozygous for *snrk1α1-3* were confirmed to be homozygous for *snrk2.2* and *snrk2.3* by genotyping with the corresponding primers. All primer sequences are provided in materials and methods.

Supplementary Figure 11



Supplementary Figure 11. SnRK2s are not required for SnRK1 activation in response to energy deficit. (A) Repression of TOR signaling in response to a sudden darkness treatment is defective in the *sesquiα2* mutant. Seedlings grown on liquid culture (0.5X MS + 0.5% sucrose) were covered 3h after the onset of the light period and samples were collected at T0, and 1h and 3h of dark treatment. TOR activity was subsequently analyzed from total protein extracts of each sample using immunoblotting and $RPS6^{S240}$ phosphorylation as readout. Graph corresponds to the average of two independent experiments (error bars, SEM). **(B)** Repression of TOR signaling in response to a sudden darkness treatment is normal in the *snrk2t* mutant. Graph corresponds to the average of 3 independent experiments (error bars, SEM; one-way ANOVA with Tukey HSD test). (*), p-value<0.05.

Supplementary Figure 12



Supplementary Figure 12. Dual effect of SnRK2.3 overexpression on primary root (PR) growth. In control conditions plants overexpressing SnRK2.3 (SnRK2.3-OE) have increased PR growth compared to the WT. Conversely, the repression of PR growth triggered by ABA is enhanced in the SnRK2.3-OE, in agreement with its known ABA hypersensitivity. Left panel, representative picture of seedlings grown vertically on 0.5X MS medium for 5d and transferred to 0.5X MS with or without ABA for 8d. Bar = 1cm. Right panels, quantification of PR length from 4 independent experiments (total number of plates: WT mock n=53, SnRK2.3-OE mock n=52, WT ABA n=48, SnRK2.3-OE ABA n=47; total number of seedlings: 47-53 per genotype and condition). (*), $p < 0.05$; (****), $p > 0.0001$ (two-tailed Student t-test).

Materials and methods

Plant material and growth

All Arabidopsis thaliana plants used in this study are in the Columbia (Col-0) background. Unless otherwise specified, plants were grown under long-day conditions (16h light, 100 µmol m−2s−1, 22°C /8h dark, 18°C) on 0.5X MS medium (0.1% MES and 0.8% phytoagar).

The sesquiα2-1 (snrk1α1-3-/- snrk1α2-1+/-) and sesquiα2-2 (snrk1α1-3-/- snrk1α2-2+/-) mutants were obtained by crossing the snrk1α1-3 (GABI_579E09) with the snrk1α2-1 (WiscDsLox320B03) and snrk1α2-2 (WiscDsLox384F5) mutants, respectively. sesquiα2 individuals were always pre-selected on BASTA-containing medium for 5-6 days together with a BASTA-resistant control line, except for germination and early development assays.

Triple snrk2.2/snrk2.3/snrk1α1-3 mutants (referred as snrk2d/α1 in the text) were obtained by crossing snrk1α1-3 to the snrk2.2/snrk2.3 double mutant (snrk2d).

Phenotype Assays

For assays of ABA sensitivity during germination and early seedling development, seeds were plated on 0.5X MS supplemented or not with ABA, and radicle emergence and cotyledon greening were computed over time under a stereoscope.

For assaying ABA sensitivity during root development, seedlings were grown vertically for 6 days in 0.5X MS (supplied with BASTA in experiments with the sesquiα2 mutant) and transferred to 0.5X MS plates supplemented or not with ABA for 8 more days. All computed parameters relate to the region of the root that developed after the transfer to mock or ABA.

Co-immunoprecipitation experiments

Interaction of SnRKs with TOR and RAPTOR

For assessing the interaction of SnRKs with TOR and RAPTOR, seedlings (proSnRK1α1::SnRK1α1-GFP, proSnRK2.2::SnRK2.2-GFP and 35S::GFP) were grown on 0.5XMS + 0.5% sucrose for 14d (7d in solid medium and 7d in liquid culture) and treated with 50 µM ABA for 40 min. GFP-tagged proteins were immunoprecipitated from whole seedling cleared protein extracts using super-paramagnetic µMAC beads coupled to monoclonal anti-GFP antibody (Miltenyi Biotec), and co-immunoprecipitated proteins were analyzed by Western blotting using anti-GFP, anti-TOR, anti-RAPTOR, anti-SnRK1α1, and anti-SnRK2 antibodies.

For immunoprecipitation of endogenous TOR, the anti-TOR antibody was coupled to Dynabeads™ Protein A (Invitrogen™) prior to its addition to the whole seedling cleared protein extracts. Co-immunoprecipitated proteins were analyzed by Western blot with anti-TOR, anti-SnRK1α1, and anti-SnRK2s antibodies.

Interaction of SnRK1 with SnRK2 and PP2CA

For assessing the interaction of SnRK1 with SnRK2 and PP2CA, seedlings (proSnRK1α1::SnRK1α1-GFP, proSnRK2.2::SnRK2.2-GFP and 35S::GFP) were grown on 0.5XMS + 0.5% sucrose for 14d (7d in solid medium and 7d in liquid culture), and roots were rapidly harvested following a 3h treatment with 50 µM ABA. GFP-tagged proteins were immunoprecipitated from cleared protein extracts using super-paramagnetic µMAC beads coupled to monoclonal anti-GFP antibody (Miltenyi Biotec), and co-immunoprecipitated proteins were analyzed by Western blotting using anti-GFP, anti-SnRK1α1, anti-SnRK2, and anti-PP2CA30 antibodies. When indicated, the SnRK1-SnRK2 interaction was analyzed also from whole seedlings following a 40 min treatment with 50 µM ABA as explained above for the interaction with TOR.

RPS6S240 phosphorylation assays

Seedlings were grown vertically for 6 days in 0.5X MS + 0.5% sucrose medium [when required, also supplemented with BASTA to allow selection of Col (B) and *sesquiα2* individuals] and transferred to 6-well plates containing 0.5X MS liquid medium supplemented with 0.5% sucrose for 6 more days (10 seedlings per 170 9.5 cm2 well containing 1 mL of medium) and treated with 50 µM ABA, 10 µM torin2 or 2 µM AZD8055 during 4h. For the ABA time course, ABA (50 µM) was added 3h after the onset of the lights and samples were collected immediately (T0) or after 15, 30, 45, 60 and 240 min. For the sudden darkness experiments, samples were collected 3h after the onset of the lights (T0) or after 1 or 3h of incubation in the dark. Samples were analyzed by Western Blot with anti-phospho-RPS6S240 and anti-RPS6 antibodies.

Custom-made SnRK1α1 and SnRK1α2 antibodies

Polyclonal Arabidopsis SnRK1α1 and SnRK1α2 antibodies were obtained by conjugating synthetic peptides (CTMEGTPRMHPAESVA and CTTDSGSNPMRTPEAGA, respectively; produced by Cocalico Biologicals, Inc. USA) to keyhole limpet hemocyanin and injecting two rabbits (performed by Cocalico Biologicals). Antibodies were affinity-purified using the original peptides linked to a SulfoLink matrix (Pierce) following instructions by the manufacturer.

Primers List:

Genotyping primers	Name	Sequence
snrk1α1-1 (Salk_127939)	snrk1α1-1-LP	ACCACACGTTGGAAACTTTTG
	snrk1α1-1-RP	ACATGAAGTGCAGATGGGTTC
snrk1α1-3 (GABI_579_E09)	snrk1α1-3-LP	CCAGCATAATAGAGAACGAAGC
	snrk1α1-3-RP	TTGACCCATCAAATAATACACGAA
snrk1α2-1 (WiscDsLox320B03)	snrk1α2-1-LP	TGCGGTTTGATGATTATAATCG
	snrk1α2-1-RP	TCGATTCCACTCCATTATTGC
snrk1α2-2 (WiscDsLox384F5)	snrk1α2-2-LP	CGTAGTGATCCACATGTGCAG
	snrk1α2-2-RP	GATTGCAGACTTTGGGTTGAG
snrk2.2 (GABI_807G04)	snrk2.2-LP	CAAGACCATACATCTGCAAGCTGG
	snrk2.2-RP	ACACCTTGATGTTTCTTCTGTGTG
snrk2.3 (Salk_107315)	snrk2.3-LP	TTGGTTTTGAGTGTTCTGCTTTTG
	snrk2.3-RP	CACCACATGACCATACATCTGCAA
T-DNA primer GABI	08409 LB (Gabi LB)	ATATTGACCATCATACTCATTGC

T-DNA primer WISC	T-DNA-Wis	AACGTCCGCAATGTGTTATTAAGTTGTC
T-DNA primer SALK	LBb1XL	ACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGG
qRT-PCR primers	**Name**	**Sequence**
RAB18	FqRAB18	TGGCTTGGGAGGAATGCTTCA
	RqRAB18	CCATCGCTTGAGCTTGACCAGA
RD29A	FqRD29A	GGAAGTGAAAGGAGGAGGAGGAA
	RqRD29A	CACCACCAAACCAGCCAGATG
DIN1	FqDIN1	CAGAGTCGGATCAGGAATGG
	RqDIN1	ATTTGACCGCTCTCACAACC
BETA1	FqKINβ1	TTATTCGCTCCTCAGGTTCC
	RqKINβ1	GGTAGGGATTCCTTGCTCTG
DRM2	FqDRM2	CTTCGACAAGCCTTCTCACC
	RqDRM2	TCGTCGCTGTATAGCCAATC
TPS8	FqTPS8	CCACAAGGTGTAAGCAAAGG
	RqTPS8	CGCGTTCTACCATTTCTCG

ACT8	FqACT8	AGTGGTCGTACAACCGGTATTGT
	RqACT8	GAGGATAGCATGTGGAAGTGAGAA
EIF4	FqEIF4	TCATAGATCTGGTCCTTAAACC
	RqEIF4	GGCAGTCTCTTCGTGCTGAC

Plant lines used in this study:

PLANT LINE	LINE ID	REFERENCE
snrk1α1-3	GABI_579E09	Mair et al., 2015 eLife
snrk1α2-1	WiscDsLox320B03	Jeong et al.,2015 BMC Plant Biol
snrk1α2-2	WiscDsLox384F5	This study
snrk2.6/ost1	SALK_008068	Mustilli et al., 2002 Plant Cell
snrk2.2/snrk2.3	GABI-Kat 807G04/SALK_107315	Fujii et al., 2007 Plant Cell
snrk2.2/snrk2.3/snrk2.6	GABI-Kat 807G04/SALK_107315/SALK_008068	Fujii et al., 2009 PNAS
proSnRK2.2::SnRK2.2-GFP	Line #2.2	Dietrich et al., 2017 Nat Plants
proSnRK1α1::SnRK1α1-GFP		Bitrián et al., 2011 Plant J
35S:SnRK2.3		Cheng et al., 2017 Plos Genetics

35S:GFP	Crozet et al., 2015 Plant J

Antibodies:

Antibody - short name	Antibody - full description	Source	Company	Product number		Dilution	Dilution buffer
Anti-SnRK1α1	Anti-SnRK1	Custom-made	This study	This study	Rabbit polyclonal	1:4000	5% w/v nonfat dry milk in 1X TBS, 0.05% Tween®
Anti-phospho-RPS6 (S240)	Anti-phospho-RPS6 (S240)	Custom-made	From Christian Meyer	Dobrenel et al. 2013 Front Plant Sci	Rabbit polyclonal	1:5000	5% w/v nonfat dry milk in 1X TBS, 0.05% Tween®
Anti-PP2CA	Anti-PP2CA	Custom-made	From Pedro Rodrigues	Wu et al. 2016 Plant Cell	Rabbit polyclonal	1:2000	5% w/v nonfat dry milk in 1X TBS, 0.05% Tween®
Anti-SnRK2s	Anti-SnRK2.2, SnRK2.3, SnRK2.6	Commercial	Agrisera	AS14 2783	Rabbit polyclonal	1:3000	5% w/v nonfat dry milk in 1X TBS, 0.05% Tween®
Anti-TOR	TOR (N) Antibody (Arabidopsis), Rabbit Polyclonal	Commercial	Abiocode	R2854-1	Rabbit polyclonal	1:1000	5% w/v nonfat dry milk in 1X TBS, 0.05% Tween®
Anti-RPS6	S6 Ribosomal Protein (54D2) Mouse mAb,	Commercial	Cell Signaling	2317	Mouse monoclonal	1:1000	5% w/v nonfat dry milk in 1X TBS, 0.1% Tween®
Anti-GFP	GFP Rabbit IgG Polyclonal Antibody	Commercial	Invitrogen	A-11122	Rabbit polyclonal	1:10000	1X TBS, 0.1% Tween®
Anti-phospho-AMPKα (T172)	Phospho-AMPKalpha Fraction (Molecular (Thr172) (40H9) Antibody Ab#1	Commercial	Cell Signaling	2535	Rabbit monoclonal	1:1000	5% w/v BSA in 1X TBS, 0.1% Tween®
Anti-RAPTOR	Raptor (A-2)	Commercial	Santa Cruz Biotechnology, Inc.	sc-518004	Mouse monoclonal	1:1000	5% w/v nonfat dry milk in 1X TBS, 0.05% Tween®

Chapter IV – Conclusions

The SnRK1 energy sensing kinase plays a crucial role in plant acclimation to adverse environmental conditions, orchestrating a vast transcriptional and metabolic reprogramming in response to the energy depletion triggered by stress. Given that most stress types compromise energy metabolism, energy depletion results as a common outcome of different non-optimal (stress) growth conditions. Therefore, SnRK1 is activated in response to a wide variety of stresses, coordinating hormonal and metabolic signaling pathways to restore energy homeostasis at the cellular and whole organism levels.

ABA is a major phytohormone that mediates physiological responses to biotic and abiotic stresses (especially drought), which are major determinants of crop losses worldwide. The activation of the ABA pathway triggers broad transcriptional changes (Nemhauser et al. 2006) that significantly overlap with those induced by activation of SnRK1 signaling (Baena-Gonzalez et al., 2007; Chapter II, Fig. 5D).

Besides regulating energy and drought stress responses, SnRK1 and ABA play a pivotal role in development, modulating several aspects of vegetative and reproductive growth, and thereby contributing to the developmental plasticity typical of higher plants (Lachowiec et al. 2016, Covarrubias et al. 2017, Mizutani et al. 2018). Processes like seed filling, embryo maturation and germination, flowering and senescence, among others, are coordinated both by ABA and SnRK1. Accordingly, manipulation of SnRK1 expression results in growth and developmental phenotypes reminiscent of altered ABA sensitivity (Radchuk et al. 2006, Jossier et al. 2009, Radchuk et al. 2010).

Despite the importance and centrality of the SnRK1 signaling pathway, our knowledge on how it is regulated and how it operates was very limited when this work was initiated. Also virtually nothing was known on whether these two related pathways, SnRK1-

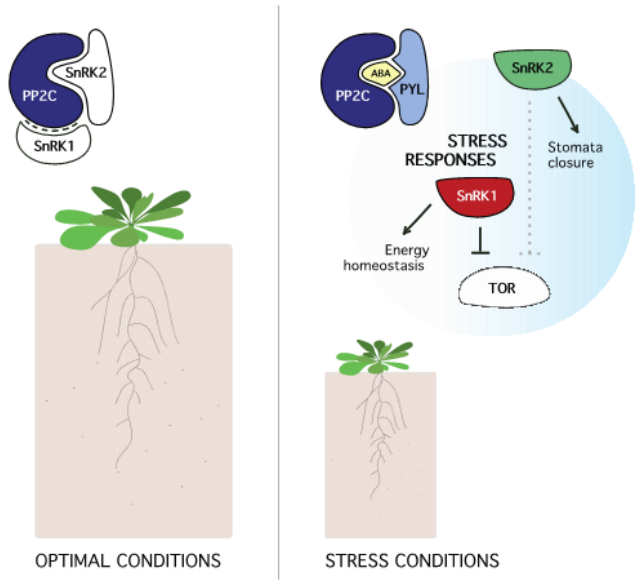
dependent energy signaling and SnRK2-dependent ABA signaling, cooperate in the orchestration of stress responses and growth or whether they instead function as independent complementary pathways. This thesis investigated potential molecular connections between ABA and SnRK1 energy signaling and the physiological outcomes in plant stress responses and growth, contributing significant insight into how SnRK1 is regulated by sugar and ABA signals, and uncovering a surprising intimate link between these two pathways that will pave the way for more studies in the future.

An initial candidate-based approach led to the discovery that the SnRK1 energy sensor is negatively regulated by the same 2C-type phosphatases that repress the ABA pathway (Chapter II). Similarly to their mechanism of action on SnRK2 kinases (Soon et al. 2012), these PP2Cs seem to block SnRK1 activity both by dephosphorylation and physical obstruction. PP2C phosphatases are required for inhibiting SnRK1 in response to sugar signals, when normal sugar levels are restored after a period of energy stress (a period of darkness in the middle of the day). However, being under PP2C regulation conferred SnRK1 the ability to respond also to ABA signals. This raised two questions: whether the PP2C-SnRK1 harboring complexes that respond to sugars are different from the PP2C-SnRK1 complexes that respond to ABA and whether the complexes responding to ABA correspond to the canonical ABA complexes that contain also SnRK2. Chapter III of this thesis provides evidence that SnRK1 is a novel component of canonical ABA complexes. Firstly, genetic data shows that SnRK2s are negative regulators of SnRK1 under mock conditions, with the *snrk2.2/2.3* (*snrk2d*) mutant displaying root growth defects (shorter primary root and reduced lateral root density) that are fully reverted by the *snrk1α1* mutation. However, in the presence of ABA the *snrk2* and *snrk1α1* mutations show additive effects, despite the fact that single *snrk1α1* mutants do not display ABA phenotypes. This indicates an involvement of SnRK2 kinases as regulators of SnRK1 and growth in ABA conditions that is functionally opposite to that in mock. Co-immunoprecipitation experiments provide further support for a direct involvement of SnRK2s in SnRK1 inhibition, revealing a clear interaction between SnRK1 and SnRK2 kinases in normal conditions that is markedly reduced in the presence of ABA. One major

outcome of this mode of regulation is the release of SnRK1 from repressor complexes in response to ABA and the concomitant inhibition of TOR and growth (post-germination growth and lateral root development). Our work demonstrated that a functional SnRK1 pathway is required for these ABA responses and that SnRK1 interacts physically with TOR, with this interaction being strongly enhanced by ABA. However, it is also clear that SnRK2 kinases are essential for repressing TOR and growth in response to ABA (Wang et al. 2018)(Chapter III). Given the fact that *in planta* no physical interaction could be detected between TOR and SnRK2s (Chapter III), the simplest explanation for the SnRK2 requirement is that these kinases are needed for providing SnRK1 the capacity to respond to ABA and to repress TOR and growth in the presence of this hormone.

Our proposed model assigns therefore a dual role for SnRK2s in the regulation of SnRK1 and growth: on one hand, in the absence of ABA, SnRK2s associate with SnRK1, forming SnRK1-inactive complexes that permit growth; in the presence of ABA, on the other hand, these SnRK2-containing complexes dissociate, releasing SnRK1 and SnRK2 kinases to drive stress responses and growth inhibition (Chapter III, Fig 4).

Figure 4



In addition to providing molecular evidence on how ABA and SnRK1 signaling are connected, the work presented in Chapter II of this thesis uncovered a novel class of negative regulators of SnRK1, PP2C phosphatases. We tested in gain-of-function approaches one representative member for each of the two subgroups that compose clade A, PP2CA and ABI1. Both phosphatases interacted physically with SnRK1 and were able to inhibit SnRK1 signaling *in vitro* and in reporter assays, supporting the idea that also other members of such clade could function as SnRK1 regulators and play partially redundant functions. In agreement with this, SnRK1 inhibition in response to sugar supply was clearly defective in quadruple *pp2c* (*Qpp2c*) mutants whilst only mild defects could be observed in a double *pp2c* mutant.

Mounting biochemical evidence shows that SnRK1 is inhibited by sugar phosphates and in particular by T6P, which operates at low concentrations compatible with its proposed hormone-like functions (Paul et al. 2008). Whether clade A PP2Cs mediate the inhibitory action of T6P (or other sugar phosphates) on SnRK1 signaling is not known, and further research is still required to clarify their potential involvement in this aspect of SnRK1 regulation. A functional correlation of T6P with glucose and ABA signaling has been revealed in Arabidopsis lines expressing *TPS1* under control of the constitutive *35S* or the seed-specific *ABI3* promoters (Avonce et al. 2004, Gomez et al. 2010). These lines display higher T6P accumulation and this is associated with glucose and ABA insensitivity, suggesting these two pathways may be connected at the level of this sugar phosphate (Avonce et al. 2004, Gomez et al. 2010). The role of PP2C in mediating SnRK1 repression by T6P could be investigated by comparing SnRK1 signaling in WT and *Qpp2c* mutant plants in response to trehalose supply, using SnRK1 marker gene expression as readout of the pathway. Trehalose feeding results in increased levels of T6P, which inhibits SnRK1 signaling in sink tissues of Arabidopsis seedlings (Delatte et al. 2011). Alternatively, the intracellular content of T6P can be perturbed through genetic manipulation of T6P synthesis using inducible overexpression of the Arabidopsis *TPS1* gene, or its bacterial homolog OtsA (Schluepmann et al. 2003, Avonce et al. 2004, van Dijken et al. 2004). The generation of a *Qpp2c* mutant in which the production of T6P is inducible in time and/or

in space (by using inducible tissue-specific promoters to drive TPS activity) would represent a valuable tool to investigate the role of clade A PP2Cs in the T6P-dependent inhibition of SnRK1.

One common paradigm of Snf1/AMPK complexes is that their activity is inhibited by a high adenylate charge (high ATP/ADP-AMP ratio). Binding of ATP to the γ subunit causes a conformational rearrangement that inhibits the interaction with activating kinases while, at the same time, favors the action of repressing phosphatases. The net result is decreased phosphorylation of the activation loop (T-loop) of the catalytic subunit and reduced kinase activity. In the case of SnRK1, T6P (and possibly other sugar phosphates) seems to functionally take over the role of adenylates in regulating kinase activity in accordance with sugar abundance. A recent study (Zhai et al. 2018), revealed that T6P is able to directly bind SnRK1α1, rendering it a poor substrate for the upstream activating kinases, SnAKs. It would be interesting to investigate whether, in analogy with adenylates in opisthokonts, T6P binding to SnRK1 positively affects the interaction with PP2C phosphatases in parallel with the reported effect in lowering the interaction with upstream kinases.

Additional differences between the plant kinase and its yeast and mammalian counterparts can be observed with regard to T-loop phosphorylation. Similarly to animals, plant SnRK1 upstream kinases (SnAKs) appear to be constitutively active (Shen et al. 2009), but in contrast to AMPK and Snf1, whose T-loop phosphorylation strongly correlates with their activity, the SnRK1α T-loop appears to be constitutively phosphorylated (Fragoso et al. 2009, Coello et al. 2012)(Chapter II). On one hand, the apparent constitutive activity could be due to technical limitations of the employed approaches. It is plausible that only a minor fraction of the SnRK1 pool is actually dephosphorylated and that immunodetection-based analyses as the ones employed in Chapter II are not sensitive enough to detect minor phosphorylation changes. Alternatively, we propose that, similarly to SnRK2, the inhibition of SnRK1 by PP2Cs is at least partly accomplished by physical blockage of the kinase, preventing its interaction

with substrates rather than provoking a major change in T-loop phosphorylation. Thus, our model proposes that SnRK1 becomes functionally active after being released from its physiological repressor(s). Release of inhibition is a common mode of activation of plant signaling pathways. Well-established examples of this are auxin and gibberellin signaling in which the transcriptional response is triggered by degradation of their respective repressors upon sensing of the hormone. Why plant systems appear to favor such mode of regulation is unclear, but potential explanations could be that activation by derepression may enable a faster response once the repressor is released, and that the constant presence of a repressor minimizes leaky signaling, ensuring that the pathway is completely inactive in the absence of its activating signals. This mode of regulation is also supported by the results of Chapter III, which show that SnRK2s are negative regulators of SnRK1 kinases in normal conditions. In normal growth conditions, plants lacking SnRK2.2 and SnRK2.3 kinases show root growth defects (shorter primary root and reduced lateral root density) that are fully reverted by the *snrk1α1* mutation. Conversely, a line overexpressing SnRK2.3 displays increased primary root length (Chapter III, Fig. S12). Furthermore, SnRK2 and SnRK1 interact physically in normal conditions but in the presence of ABA this interaction is broken, suggesting that SnRK2s are directly involved in the repression of SnRK1 in the absence of ABA. Our results hence point that, in the absence of ABA, SnRK1 is kept in an inactive state by the physical interaction with the ABA-dependent SnRK2s (i.e., SnRK2.2, SnRK2.3) which therefore act as repressors of SnRK1 in normal conditions. Interestingly, Q*pp2c* mutants have, likewise the *snrk2d* mutant, reduced primary root length (Chapter II; Fig. S5B-C). This, together with the fact that PP2Cs are also involved in the activation of SnRK1 by ABA (Chapter II) and that the interaction between SnRK1 and PP2CA is, similarly to the SnRK1-SnRK2 and SnRK2-PP2C interactions, markedly reduced in ABA (Chapter III), suggests that the three proteins could be part of the same complex. To assess this, the interaction between SnRK1 and SnRK2 or PP2C proteins could be tested in co-immunoprecipitation experiments in Q*pp2c* or *snrk2d* mutant lines, respectively. If PP2Cs are required to mediate the interaction between SnRK1 and SnRK2, then a quadruple *pp2c* mutation would reduce or abrogate such interaction. The same rationale can be applied for using a *snrk2d* mutant to test the role

of SnRK2s in the formation of SnRK1-PP2C complexes. A more quantitative approach would be to perform isothermal titration calorimetry (ITC) experiments on recombinant proteins. Eventually, the SnRK1-SnRK2-PP2C complexes could be visualized *in vivo* by immune transmission electron microscopy coupling the corresponding antibodies (SnRK1α1, SnRK1α2, *Snrk2d*/2.6 and PP2CA) with three different sizes of gold particles.

Our findings show that the interaction among SnRK1, SnRK2s and PP2CA is disrupted upon treatment with ABA, suggesting that the inhibitory effect of SnRK2s (and PP2CA) on SnRK1 could serve as a mechanism to avoid leaky SnRK1 signaling under optimal growth conditions (i.e., in absence of ABA). In this scenario, the growth defect of the *snrk2d* and Q*pp2c* mutant lines could be explained by an overactivation of SnRK1 signaling and, probably, by a consequent partial inhibition of the growth-promoting TOR kinase (see below).

When investigating the interaction between SnRK2s and SnRK1, the immunoprecipitation of SnRK2.2-GFP from transgenic Arabidopsis plants retrieved readily SnRK1α1, but none of the endogenous SnRK2s detected by the employed antibody (SnRK2.3, SnRK2.3 or SnRK2.6 - Chapter III, Fig S8A), supporting the idea that SnRK1 and SnRK2s form complexes in a 1:1 ratio. Furthermore, as explained above, it is likely that PP2C phosphatases, interacting both with SnRK1 and SnRK2s (Chapter II and Chapter III of this thesis), could be part of the same supramolecular complexes. Definitely, more experiments are needed to clarify in depth the nature of the SnRK2-PP2Cs-SnRK1 complexes in terms of the stoichiometry of components and the specificity of SnRK2 (and PP2C) proteins associated with SnRK1.

SnRK1α has been shown to be present in complexes of very different size (from 65 to 570 KD), with SnRK1 activity being concentrated in the lower molecular weight range roughly corresponding to single SnRK1 trimers (Nunes et al. 2013). This lends support to the existence of complexes where SnRK1 activity is suppressed ("SnRK1 repressor complexes") and prompts a role for SnRK2 kinases in the formation of these complexes of high molecular weight and low SnRK1 activity.

In order to examine whether SnRK2s play merely a "structural" role in SnRK1 regulation, it would be useful to generate transgenic lines complementing the *snrk2d* mutant with a catalytically inactive version of either of the two kinases driven by its endogenous promoter (*proSnRK2.2::SnRK2.2*^{K52N}, *proSnRK2.3::SnRK2.3*^{K51N}). The complementation of the phenotypes in control (reduced primary root growth and lower lateral root density than wild type – Chapter III Fig. 3C) and ABA conditions (higher ABA insensitivity in root emergence and cotyledon greening than wild type – Chapter III Fig. 3A-B) would reveal if both functions (positive and negative regulation of growth) are dependent on SnRK2 kinase activity. A similar complementation approach (including *proSnRK2.6::SnRK2.6*^{K50N}) could also be applied to the triple *snrk2.2/snrk2.3/snrk2.6 (snrk2t)* mutant to further clarify whether TOR inactivation in response to short-term ABA treatment requires SnRK2 kinase activity (Wang et al. 2018)(Chapter III, Fig. S6B).

The experiments in which the activation status of TOR was monitored in response to different stimuli revealed that SnRK1 mediates TOR inhibition in response to ABA and energy deficit (i.e., dark treatment; Chapter III, Fig 1C – S11A). On the contrary, the same analysis in the *snrk2t* mutant (Chapter III, Fig. S11B) demonstrates that, while SnRK2s are fully required to inhibit TOR in response to ABA (Wang et al. 2018), they are dispensable for inhibiting TOR in response to energy deficit (Chapter III, Fig. S6B - S11B). These observations suggest the existence of SnRK1 complexes that respond to stimuli other than ABA (e.g. energy deficit) and that are probably formed independently of group III SnRK2s. Given that clade A PP2Cs are required both for the sugar-dependent and ABA-dependent regulation of SnRK1 (Chapter II of this thesis), it is possible that PP2Cs are present in both types of SnRK1 complexes. One possible way to support this hypothesis would be to test the ability of the Q*pp2c* mutant to repress TOR in response to energy depletion as well as its ability to induce TOR re-activation in response to sugar supplementation after stress.

In addition to providing significant insight on how SnRK1 is regulated, the work presented in this thesis has contributed to deciphering how the crosstalk between sugar and ABA signaling is achieved and how it affects plant growth and development. TOR is a positive master regulator of growth, which catalyzed the interest of the plant scientific community during the last decade. The action of TOR is functionally counteracted by SnRK1, which, on the contrary, operates as a growth inhibitor. Both TOR and SnRK1 pathways are essentially conserved among eukaryotes (Roustan et al. 2016) with few exceptions in plants mainly related to specific regulatory aspects, or particular components (e.g. the absence of Rictor in plant genomes, or the plant-specific SnRK1βγ regulatory subunit). In plants, the regulation of TOR and SnRK1 by sugars seems to play a prominent role compared to other systems. Mammalian mTOR, for example, is highly regulated by essential amino acids (Bar-Peled et al. 2014) while, in yeast and mammals, SnRK1 orthologues respond to variations in the adenylate charge (Chandrashekarappa et al. 2011, Oakhill et al. 2011). In plants, sugar levels directly regulate TOR and SnRK1 in an opposite fashion: TOR signaling is activated by photosynthesis-derived glucose, whereas SnRK1 is inhibited by glucose, sucrose, and various sugar phosphates (Baena-Gonzalez et al. 2007, Zhang et al. 2009, Nunes et al. 2013, Xiong et al. 2013, Li et al. 2016). The reported mechanism of TOR activation in response to glucose (Xiong et al. 2013) is likely to be at least partly mediated by the inhibition of SnRK1 which is able to phosphorylate and inactivate TOR during energy starvation (Nukarinen et al. 2016)(and Chapter III).

TOR and SnRK1 are also oppositely regulated by ABA, functionally mirroring the action of sugars on these growth regulators. Our work demonstrated that SnRK1 is activated by ABA through the disruption of inhibitory interactions with PP2C phosphatases (Chapter II) and SnRK2 kinases (Chapter III). Whilst SnRK2s are necessary for repressing TOR in response to ABA (Wang et al. 2018) (Chapter III), TOR is also involved in the regulation of ABA signaling through phosphorylation of the PYR/PYLs receptors. The phosphorylation of the PYR/PYLs at conserved residues lowers their affinity for ABA, thereby limiting their activation and establishing a positive feedback loop on TOR activation. This feedback mechanism is important to avoid leaky ABA signaling under nutrient-rich conditions and

to desensitize ABA receptors once the energy levels are sufficiently high to cope with the stress condition (Wang et al. 2018).

The reliance of the ABA-dependent TOR inactivation on SnRK1 (Chapter III) provides a molecular explanation for the attenuation of ABA phenotypes by sugars sometimes observed in the literature. For example, in wild type seedlings the extent of primary root length inhibition by 5 µM ABA observed in this thesis (Chapter III, no sugar in the medium) is similar to that reported for 50 µM ABA in medium containing 3% sucrose (Fujii et al. 2007). It is tempting to speculate that this sugar-dependent desensitization to ABA relates to the inhibitory effect that sugars have on SnRK1 activity. In the light of our results, several scenarios can be envisioned to explain this at the molecular level. Sugars could promote the formation of SnRK1-repressor complexes containing PP2Cs and SnRK2s, thereby counteracting the effect of ABA on SnRK1/SnRK2 release and activation. Alternatively, sugars could promote the formation of other type of complexes, unable to respond to ABA, that compete with SnRK2s for SnRK1 and PP2Cs. A third possibility is that the effect of sugars relates to the desensitization to ABA: activation of TOR by sugars would trigger the phosphorylation and inactivation of the PYR/PYL ABA receptors and hence block the first step of ABA signaling. These not mutually exclusive hypotheses could be tested employing various biochemical and phenotypic approaches. A first set of co-immunoprecipitation experiments combining sugar and ABA could be undertaken in wild type Arabidopsis seedlings. If sugars directly, or indirectly, promote the formation of SnRK1-SnRK2-PP2C complexes, one would expect that sugar enhances SnRK1-SnRK2 and SnRK1-PP2C interactions. If so, it would be informative to monitor TOR pathway activation in response to sugar supplementation using RPS6 protein phosphorylation as read-out. The counteracting effect of sugar on ABA signaling could also be assessed with this experimental set-up by comparing TOR activity in response to ABA, sugar and a combination of ABA and sugar. To assess whether SnRK2s are required for the sugar-mediated suppression of SnRK1, TOR activity could be monitored also in the *snrk2t* mutant. If SnRK2s play a role in this, the expectation would be that sugar provision would

induce TOR activation to a lower extent in *snrk2t* than in WT seedlings. The same approach could be also applied to higher order *snrk2* mutants (Fujii et al. 2011).

The possibility of sugars favoring the formation of other SnRK1 complexes (not responding to ABA) could be tested by analyzing the SnRK1 interactome through mass spectrometry from seedlings treated with ABA, sugar, and both. Finally, the contribution of the above mentioned mechanism of PYR/PYL receptor inactivation could be evaluated using transgenic PYR/PYL knock out lines (Zhao et al. 2018) complemented with a PYR/PYL receptor mutated in the residue phosphorylated by TOR (e.g. PYL1-S119A)(Wang et al. 2018) and analyzing the root growth phenotype in ABA in presence of sugars. This line is defective in the TOR-mediated desensitization of ABA signaling (Wang et al. 2018) and therefore a reduction of the sugar-mediated attenuation of the ABA phenotype is expected in case the hypothesis is correct.

Another interesting aspect correlating ABA with growth is the fact that, in several plant species, ABA can function as a growth promoter instead of a growth repressor when provided at low dosages (Humplik et al. 2017). In Arabidopsis a growth-promoting effect of ABA has been reported for concentrations ranging from 10 to 500 nM (Fujii et al. 2007, Dietrich et al. 2017, Salem et al. 2018). It is reasonable to assume that the TOR-mediated inactivation of ABA receptors avoids the activation of the canonical ABA pathway at low dosage, being thereby a prerequisite for ABA-mediated growth promotion. Accordingly, mutants impaired in the TOR pathway are hypersensitive to ABA (Kravchenko et al. 2015, Salem et al. 2017, Wang et al. 2018). Noteworthy, both inhibition and promotion of growth induced by ABA seems to be dependent on SnRK2.2 and SnRK2.3 kinases, as the double knock out line for these genes fails to respond to the hormone for both of the outcomes in primary root length (Fujii et al. 2007, Dietrich et al. 2017)(Chapter III). Whereas the function of SnRK2s in response to growth-inhibiting ABA concentrations is well established, little is known about their role in the presence of lower, growth-promoting, ABA concentrations. It is reasonable to think that, similarly to what is reported in Chapter III in control conditions, SnRK2 proteins could work as repressors of

SnRK1, releasing TOR from inactivation and thus resulting in growth promotion. More experiments are needed to elucidate the relations between SnRK1 and SnRK2s in response to low dosage ABA. One easy-to-test prediction would be that low ABA concentrations are not sufficient to trigger the dissociation of SnRK1-SnRK2 complexes reported in Chapter III.

It is therefore possible that the dual role of SnRK2s in regulating growth in control and ABA conditions is related to the opposite effect that ABA has on growth at low and high concentrations. As a matter of fact, Arabidopsis mutant lines impaired in ABA synthesis and perception display reduced growth in control conditions (Barrero et al. 2005, Dietrich et al. 2017, Zhao et al. 2018), indicating that in normal conditions basal ABA signaling acts to promote growth.

The results presented in Chapter III indicate that the effect of SnRK2s on growth regulation is mediated by SnRK1, suggesting a new regulatory role of SnRK2s on SnRK1 signaling. It would be interesting to explore whether, in addition to its role in mediating high-ABA growth inhibition, SnRK1 plays a role also in this type of low-ABA response. The type of SnRK2-dependent regulation of SnRK1 proposed in this thesis invokes a "structural" role for SnRK2s that is compatible with the very low ABA concentrations used in some studies (Humplik et al. 2017, Salem et al. 2018). To our knowledge, no evidence for SnRK2 kinase activation was reported for such low hormone concentration (e.g. 10 nM ABA). One possible way by which, in low dosage, ABA could inhibit SnRK1 could be by increasing SnRK2 levels *via* transcriptional and/or translational regulation, protein stabilization or a combination of those. Finally, it is conceivable that the potential suppression of SnRK1 activity in low ABA would lead to growth through the activation of TOR signaling.

Supporting this line of thought, it was recently reported that ABA-activated SnRK2 kinases (SnRK2.2, SnRK2.3 and SnRK2.6) play a role in regulation of energy metabolism of Arabidopsis leaves in non-stress conditions (Yoshida et al. 2019). Metabolite profiling revealed a strong flux increase in the TCA cycle in the *snrk2t* mutant, suggesting an overall

negative regulatory role in mitochondrial respiration for these components (Yoshida et al. 2019). Whether in these conditions the role of SnRK2s as inhibitors of the TCA cycle is mediated by SnRK1 is not known, but, in the light of our data presented in Chapter III, this hypothesis would be worth testing. In addition, the *snrk2t* mutant accumulates higher levels of trehalose (Yoshida et al. 2019). Although T6P levels appear normal, it is still possible that other branches of sugar metabolism are altered in this mutant, inhibiting SnRK1 directly or indirectly, and thus resulting in growth promotion.

In summary, the work presented in this dissertation reveals new mechanisms of SnRK1 regulation by components of the ABA pathway. Both clade A PP2C phosphatases and ABA-activated SnRK2 kinases (negative and positive regulator of ABA pathway, respectively) act as repressors of SnRK1 signaling in non-stress conditions. Several lines of evidence presented in Chapter II and III support the idea that the activation of SnRK1 is achieved by derepression in response to energy depletion and high exogenous ABA. A model is proposed in which the ability of SnRK1 complexes to respond to different stimuli is conferred by the different molecular partner(s) associated with SnRK1. Members of clade A PP2C phosphatases are likely to take part in ABA-responsive SnRK1 repressor complexes and in the formation of repressive complexes in response to sugars, whilst class III SnRK2s are required for the formation of ABA-responsive SnRK1 complexes but seem to be absent from complexes that respond to energy stress. In this model (Chapter III – Fig. 4) ABA-activated SnRK2s play a dual role in growth control by differential regulation of SnRK1. In optimal conditions, SnRK2-SnRK1-PP2Cs complexes are stably formed, preventing SnRK1 from interacting with downstream effectors; in the presence of ABA, these complexes dissociate via canonical ABA signaling, allowing the release of SnRK1 and the subsequent phosphorylation of its targets (e.g. TOR). In the absence of SnRK2s the SnRK2-SnRK1-PP2C complexes are not formed and the corresponding mutants cannot activate SnRK1 is response to ABA. Further analyses of the *snrk2t* and Q*pp2c* mutants (using the strategies employed in Chapters II and III for Q*pp2c* and *snrk2d*, respectively) would be essential to conclusively establish which components are required

for activation of SnRK1 in response to energy deficit and ABA and for repression of SnRK1 in response to sugars.

Plant growth is regulated by two functionally opposite growth regulators, the energy-sensing SnRK1 and the nutrient-sensing TOR kinases. The concerted action of SnRK1 and TOR defines the final growth outcome dictated by the environment. The ability to modify growth in response to different conditions is at the heart of the developmental plasticity typical of plants. By linking two conserved and interconnected master regulators of growth with components of the ABA pathway, the work of this thesis contributes to our understanding of the molecular events that drive plasticity in response to low energy and high ABA. Both signals converge on SnRK1 activation and (at least in the short term) result in the repression of TOR activity in a SnRK1-dependent manner. Interestingly, members of the "core" ABA pathway (namely, PYR/PYLs, PP2Cs and SnRK2s) are deeply embedded in the complex network of SnRK1 regulation, releasing SnRK1 from inhibition in response to both ABA and low energy, and keeping it functionally inactive under normal conditions.

Land plants (embryophytes) have a monophyletic origin indicating that they are descendent from a common ancestor that started colonizing terrestrial habitats around 500 million years ago (Kenrick et al. 1997, Morris et al. 2018). A progressive independence from water can be traced during embryophytes evolution from bryophytes (mosses) to spermatophytes (gymnosperms and angiosperms) regarding vegetative growth and reproduction. Plants evolved several strategies to cope with water deficit, ranging from desiccation tolerance in vegetative (e.g. bryophytes) or reproductive tissues (e.g. pollen and seeds) to the development of mechanisms that allow the maintenance of a relatively constant, water content (e.g. control of transpiration rate, osmotic adjustment, and others). ABA plays a critical role in establishing desiccation tolerance and in inducing cellular and whole-plant adjustments to tolerate water scarcity, including developmental arrest in several plant tissues and developmental stages [e.g. pollen and seeds (Meurs et al. 1992, Chibi et al. 1995, Raz et al. 2001, Nakashima et al. 2013, Dai et

al. 2018, Pacini et al. 2019). Being necessary for processes that were essential for land colonization, the acquisition of ABA signaling represents therefore a fundamental trait of the evolutionary success of spermatophytes, and in particular angiosperms.

The core module of ABA signaling (i.e. class III *SnRK2*, clade A *PP2C*, *PYR/PYL*) is highly conserved in land plants (Hauser et al. 2011, Sakata Y. et al. 2014), being first identifiable in bryophytes (Hauser et al. 2011, Takezawa et al. 2011, Wang et al. 2015), and suggesting that this acquisition played an important role in the water to land transition. Functional studies in the moss *Physcomitrella patens* demonstrate that SnRK2s and PP2Cs are major regulators of vegetative desiccation tolerance (Komatsu et al. 2013, Shinozawa et al. 2019) which is considered one of the first evolutionary requirements for growth outside water (Oliver et al. 2000). Current evolutionary models report that ABA was acquired in bryophytes to finely control a pre-existing mechanism of dehydration tolerance depending on PP2C and SnRK2 proteins (Komatsu et al. 2013, Shinozawa et al. 2019).

On the other hand, the unicellular green algae *Chlamydomonas reinhardtii* lacks clade A PP2Cs and PYR/PYL receptors but harbors both SnRK2 and SnRK1 (Hauser et al. 2011, Colina et al. 2019). SnRK2s probably originated by a gene duplication event of a *SnRK1α* gene, and acquired functions in responses to salt/osmotic stress, nitrogen or sulfur limitation (Gonzalez-Ballester et al. 2008, Valledor et al. 2014, Colina et al. 2019, Jamsheer et al. 2019).

While SnRK2 proteins are exclusive to the plant lineage, both SnRK1 and TOR are evolutionary conserved among all eukaryotes (with the exception of some intracellular parasites), indicating that the nutrient sensing function of the SnRK1-TOR axis is deeply rooted in the tree of life (van Dam et al. 2011, Hardie et al. 2012, Roustan et al. 2016). However, in addition to the highly conserved nutrient-sensing capacity, the SnRK1/AMPK-TOR axis acquired other mechanisms of regulation in different lineages to fit specific growth patterns and lifestyles. For example, mammalian AMPK is activated by leptin to stimulate feeding behavior when blood glucose levels are low (Hardie et al. 2012, Wang

et al. 2018). In higher plants, both TOR and SnRK1 have been implicated in the response to most phytohormones, but only in a few cases the responses have been unraveled at the molecular level [reviewed in (Jamsheer et al. 2019)]. The results of this thesis suggest that, in the plant lineage, the ancient SnRK1/AMPK-TOR axis evolved to recognize ABA signals by recruiting core ABA signaling components (SnRK2s and PP2Cs) as SnRK1 regulators. This provided the SnRK1-TOR axis the capacity to regulate growth in accordance with water availability, potentially boosting the colonization of terrestrial habitats. Given that the evolution of SnRK2s precedes their involvement in ABA signaling it would be interesting to investigate when their role in SnRK1 regulation arose.

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