Regulation of the shoot apical meristem by metabolic signals and the SnRK1 protein kinase

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Resumo

As plantas vão produzindo novos órgãos ao longo de toda a sua vida. Isto depende de células estaminais pluripotentes que residem em microambientes chamados de meristemas. O meristema apical caulinar é responsável pelo desenvolvimento de todos os tecidos e órgãos produzidos acima do nível do solo, sendo responsável por manter o equilíbrio entre proliferação e diferenciação celular para permitir a produção contínua de órgãos como folhas, flores e caules.

A estrutura do meristema é altamente organizada em zonas distintas, incluindo a zona central no topo do meristema, onde as células estaminais estão localizadas, uma zona periférica onde os órgãos laterais são iniciados e o centro organizador, que desempenha um papel central na manutenção das células estaminais. A regulação das diferentes zonas no meristema é mantida principalmente pela comunicação e coordenação entre diferentes fatores de transcrição e diferentes vias de sinalização hormonal. No fim, estas interações irão influenciar o crescimento e a diferenciação celular na planta.

O crescimento é um processo extremamente exigente em termos energéticos, que está intimamente relacionado com o ciclo diurno e é limitado por condições ambientais desfavoráveis que comprometam a fotossíntese e/ou respiração, levando a uma redução nos níveis de ATP. O crescimento é, portanto, finamente regulado por sistemas que detetam flutuações nos níveis de energia do organismo, de tal modo que a incapacidade de ajustar as taxas de crescimento aos recursos disponíveis irá refletir-se negativamente na aptidão e sobrevivência do organismo. No entanto, ainda não se compreende totalmente como o estado energético da planta influencia a atividade do meristema, regulando deste modo o crescimento diferencial da mesma.

O déficit energético provocado, por exemplo, pelo stress promove a ativação da quinase Sucrose Non-Fermenting 1 (SNF1) - related kinase1 (SnRK1), o ortólogo vegetal da quinase de levedura SNF1 e da quinase animal AMP-activated kinase (AMPK). Uma vez ativada, a SnRK1 inicia vastas mudanças a nível transcricional e metabólico para induzir processos catabólicos produtores de energia e reprimir processos anabólicos consumidores de energia, com o objetivo final de restaurar a homeostase energética. Esta gestão de recursos mediada pela SnRK1 é crucial para lidar com situações de stress; além disso, esta quinase desempenha igualmente um papel regulador em vários processos durante o desenvolvimento da planta. Isso faz com que se tenha atribuído à SnRK1 funções importantes na plasticidade do desenvolvimento da planta, permitindo modificações na sua arquitetura e nas transições de desenvolvimento, de acordo com as condições ambientais.

Nesta tese, demonstra-se a importância dos fatores ambientais, como a luz, para a função do meristema. Por um lado, os dados mostram que a luz atua indiretamente através do seu efeito na fotossíntese e na produção de açúcares, promovendo a acumulação de um regulador chave do meristema, o fator de transcrição SHOOT MERISTEMLESS (STM). Adicionalmente, demonstra-se que o sensor de energia SnRK1 é essencial na regulação da resposta de STM à luz e desempenha um papel duplo no meristema apical. Em condições energéticas desfavoráveis, a ativação da SnRK1 limita a acumulação da proteína STM, provavelmente como um meio de regular negativamente o crescimento. Por outro lado, o silenciamento de SnRK1 no meristema leva a graves defeitos na organização e função do meristema, sugerindo que SnRK1 desempenha aqui também um papel importante na, contribuindo para a integridade do meristema.

Adicionalmente, nesta tese demonstra-se que os principais reguladores do meristema, STM e outros fatores de transcrição associados, como BEL1-like 8 (BLH8), BLH9, ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1) e WUSCHEL (WUS), interagem com a subunidade catalítica de SnRK1, SnRK1α1. Em complemento a esta interação e apoiando uma conexão funcional entre SnRK1 e estes fatores de transcrição, em ensaios com protoplastos de mesófilo de Arabidopsis, SnRK1 demonstrou exercer um forte efeito inibitório na atividade de STM, potencialmente através da fosforilação direta de STM e/ou dos seus parceiros.

Em suma, esta tese identifica um mecanismo pelo qual a informação proveniente de fatores ambientais converge na regulação da atividade do meristema apical caulinar através de sinalização mediada por açúcares e pela proteína quinase SnRK1.

Abstract

Plants form new organs throughout their lifetime. This depends on the activity of pluripotent stem cells that reside in microenvironments called meristems. The shoot apical meristem (SAM) is responsible for the development of all above-ground tissues, keeping a fine balance between cell proliferation and differentiation, to enable the continuous production of organs such as leaves, flowers, and the stem.

The structure of the SAM is highly organized into distinct layers and zones. The central zone (CZ) harbours the stem cells that feed the peripheral zone (PZ), where new organ primordia are initiated, and the rib zone (RZ), which forms the stem. The RZ is beneath the CZ and harbours the organizing centre (OC), which is crucial for stem cell maintenance. The regulation of the different zones in the SAM is maintained mainly by the communication and coordination of transcriptional networks and hormone signalling pathways. Ultimately these interactions will impact growth and differentiation.

Growth is a highly energy-demanding process that is tightly linked to the diurnal cycle and is limited by unfavourable environmental conditions that compromise photosynthesis and/or respiration and deplete the ATP pool. Growth is therefore tightly regulated by systems that sense fluctuations in energy availability, and the inability to adjust growth rates to the available resources has a penalty on fitness and survival. Nevertheless, how the energy status of the plant influences meristem activity to modulate growth is poorly understood.

Energy deficit conditions promote the activation of Sucrose Non-Fermenting 1 (SNF1)-related kinase1 (SnRK1), the plant ortholog of yeast SNF1 and mammalian AMP-activated kinase (AMPK). Upon activation, SnRK1 drives a vast transcriptional and metabolic reprogramming to induce

energy-producing catabolic processes and repress energy-consuming anabolic processes, with the ultimate aim of restoring energy homeostasis. This SnRK1-mediated management of resources is crucial for coping with stress but also has an impact on multiple aspects of plant development. This suggests that SnRK1 plays a role in plant developmental plasticity, enabling modifications in plant architecture and developmental transitions in accordance with the environment.

In this thesis, I demonstrate the importance of light, for SAM function. I show that light acts indirectly through its effect on photosynthesis and sugar production, promoting the accumulation of a key regulator of meristem function, the SHOOT MERISTEMLESS (STM) transcription factor. I further show that the SnRK1 energy sensor is implicated in the regulation of STM in response to light and that it plays a dual role in the SAM. Under unfavourable conditions, strong activation of SnRK1 limits STM protein accumulation, probably as a means to downregulate growth. Under favourable conditions, silencing of SnRK1 in the meristem leads to severe defects in meristem organization and function, suggesting that SnRK1 is also necessary for meristem organization and function, acting as a keeper of meristem integrity. I further show that the SnRK1 catalytic subunit, SnRK1α1, interacts with STM and with its partner transcription factors BEL1-like 8 (BLH8), BLH9, ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1), and WUSCHEL (WUS). A functional connection between SnRK1 and these key SAM regulators is further supported by reporter assays in Arabidopsis mesophyll protoplasts, where SnRK1 exerts a strong inhibitory effect on STM activity. This inhibition requires SnRK1 kinase activity and potentially involves the direct phosphorylation of STM and/or its partners.

Altogether, this thesis uncovered a mechanism through which environmental information may be translated into modified SAM activity *via* sugar signals and the SnRK1 protein kinase.

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List of Abbreviations

4-Cl-IAA	4-chloro-indole-3-acetic acid
ABA	abscisic acid
AHK	ARABIDOPSIS HISTIDINE KINASE
AHP	ARABIDOPSIS HISTIDINE PROTEINS
amiRNA	artificial microRNA
ARF5 / MP	AUXIN RESPONSIVE FACTOR 5 / MONOPTEROS
ARR	ARABIDOPSIS RESPONSE REGULATOR
AS1/2	ASYMMETRIC LEAVES 1/2
ATH1	ARABIDOPSIS THALIANA HOMEOBOX GENE1
AUX/LAX	AUXIN1/LIKEAUXIN1
BELL	BEL1-like
BP	BREVIPEDICELLUS / KNAT1
CBM	carbohydrate binding module
CBS	cystathionine β-synthase
CLV3	CLAVATA 3
CK	cytokinin
CKX	CYTOKININ OXIDASE
CRF	CYTOKININ RESPONSE FACTOR
CRN	CORYNE

COP1	CONSTITUTIVE PHOTOMORPHOGENIC 1
CZ	Central Zone
DET1	DEETIOLATED1
FLC	FLOWERING LOCUS C
HAM	HAIRY MERISTEM
HD	homeodomain or homeobox
HL	high light
IAA	indole-3-acetic acid
IM	inflorescence meristem
iP	N6-isopentenyl-adenine
IPT	ISOPENTENYL TRANSFERASE
KNOX	KNOTTED1-like homeobox
LL	low light
LOG	LONELY-GUY
NAP	nuclear auxin pathway
NMT1	N-MYRISTOYLTRANSFERASE 1
OC	Organizing Centre
PAA	phenylacetic acid
PNF / BLH8	POUNDFOOLISH / BEL1-LIKE HOMEODOMAIN 8
PIN	PIN-FORMED

PNY / BLH9	PENNYWISE / BEL1-LIKE HOMEODOMAIN 9 / BELLRINGER (BL
	REPLUMLESS (RPL) / LARSON (LSN) / VAAMANA (VAN)
RAM	Root Apical Meristem
RZ	Rib Zone
SAM	Shoot Apical Meristem
SAW1 / BLH2	SAWTOOTH1
SAW2 / BLH4	SAWTOOTH2
SnAK	SnRK1-Activating Kinase
SnRK1	Sucrose Non-Fermenting 1 (SNF1)-related kinase 1
STM	SHOOT MERISTEMLESS
TALE	Three-amino acid length extension
TOR	TARGET OF RAPAMYCIN
Tre6P	Trehalose 6-phosphate
TUB	TUBULIN
tZ	trans-zeatin
WUS	WUSCHEL
Y2H	Yeast two-hybrid

CHAPTER I

General Introduction

1.1. Plant development and meristems

Plants are characterized by their ability to grow and develop continuously throughout their lifetime. This contrasts with animals, where morphogenesis occurs mostly during embryogenesis, with some further allometric changes occurring during juvenile growth (Heidstra and Sabatini, 2014). Postembryonic development is key to plant´s fitness and survival. By being usually fixed in a given area, plant adaptation to different environmental conditions relies largely on growth modifications and subsequent changes in architecture and physiology. This plasticity is possible due to the existence of meristems, which are organized structures of undifferentiated cells that undergo constant cell proliferation to produce new organs (Barton, 2010; Kitagawa and Jackson, 2019).

In the meristems, stem cell proliferation and differentiation is dynamically balanced, ensuring that the number of stem cells and of their dividing daughter cells remains constant, despite the continuous incorporation of differentiating cells into new organs (Gaillochet and Lohmann, 2015; Schwartz et al., 2021).

There are two apical meristems in the mature embryo: the shoot apical meristem (SAM) and the root apical meristem (RAM). The SAM is responsible for producing all of the above-ground organs whereas the RAM produces the entire root system (Heidstra and Sabatini, 2014).

Additional meristems and specific cell types with pluripotent competences add complexity to the various organs and thus to plant architecture. For example, axillary meristems (AMs), located in the axils of leaves, are responsible for the formation of branches, contributing to the development of the longitudinal axis of the plant (Long and Barton, 2000). On the other hand, a secondary meristem, the procambium, allows plants to grow radially,

contributing to the development of the vascular system and providing mechanical support (Larson, 1976; Schwartz et al., 2021).

Later in development, there are three types of SAM, the vegetative meristem, that produces leaves before the floral transition, the inflorescence meristem (IM), that produces floral buds after the transition, and the floral buds that are meristems themselves and produce floral organs (Chang et al., 2020).

The SAM of dicot plants, such as *Arabidopsis thaliana* (Arabidopsis), comprises three distinct cell layers. The L1 and L2 are the outer cell layers (tunica), that give rise to the epidermal and subepidermal layers, respectively, through anticlinal cell divisions (where the division plane is perpendicular to the surface). The L3 layer resides in the interior of the meristem and gives rise to the stem and vascular tissues through variable patterns of division (Lopes et al., 2021; Shi and Vernoux, 2019; Steeves, 2006).

Additionally, the SAM can be subdivided into three well-characterized functional domains: the central zone (CZ) which harbours the stem cell niche, the peripheral zone (PZ) which contains daughter cells of those in the CZ and which will contribute to the formation of new organ primordia, and the rib zone (RZ) which contributes to the formation of the vasculature and of the components of the stem (Lopes et al., 2021; Reinhardt, 2003)(Figure 1). Underneath the CZ, there is also a small population of cells called the organizing centre (OC), which acts to control the activity of the stem cells in the CZ. Interestingly, the cell division rate of the CZ is very low, minimizing the likelihood of mutations produced by individual stem cells that would impact large sectors of the aerial organs of the plant. By contrast, the cell division rate increases markedly at the meristem periphery, where new primordia form by incorporating cells from all layers (Laufs et al., 1998; Reddy et al., 2004; Truskina and Vernoux, 2018).

The different regions of the SAM are characterized by the expression of key regulatory genes, such as *CLAVATA3* (*CLV3*) that marks the stem cell niche in the CZ (Fletcher, 1999), and *WUSCHEL* (*WUS*), encoding a homeodomain transcription factor whose expression defines the OC (Mayer et al., 1998).

In Arabidopsis, SAM homeostasis is finely regulated by a negative-feedback loop between WUS and CLV3. This signalling pathway has evolved as a central regulatory mechanism to coordinate stem cell proliferation and differentiation in the SAM (Brand et al., 2000; Lopes et al., 2021; Schoof et al., 2000). The WUS protein moves upward from the OC into the CZ through plasmodesmata (Daum et al., 2014) where it promotes stem cell proliferation and represses differentiation. In addition, WUS promotes *CLV3* expression through direct binding to *CLV3* promoter. In turn, stem cells secrete the small CLV3 peptide into the intercellular space, where it is perceived by membrane receptors, such as CLAVATA1 (CLV1), CLAVATA2 (CLV2), and CORYNE (CRN) receptors, which in turn signal in the OC to repress *WUS* expression (Clark et al., 1997; Lopes et al., 2021). Accordingly, *clv3* loss of function mutations lead to enlarged meristems and enhanced organ production due to the lack of inhibition of *WUS* expression (Diévart et al., 2003; Fletcher, 1999). This interaction between CLV3 and WUS establishes a negative feedback regulatory loop that contributes to stem cell homeostasis in the SAM (see chapter 2 for further details).

Besides the WUS-CLV3 regulatory loop, a balance between stem cell division and differentiation is achieved through the coordinated crosstalk between different phytohormones, in particular cytokinins (CKs) and auxin. CKs stimulate cell proliferation (Schaller et al., 2014), whilst auxin promotes cellular differentiation and organ outgrowth (Schaller et al., 2015).

Accordingly, maintaining the balance between auxin and CK is a key feature for stem cell regulation and thus, organogenesis.

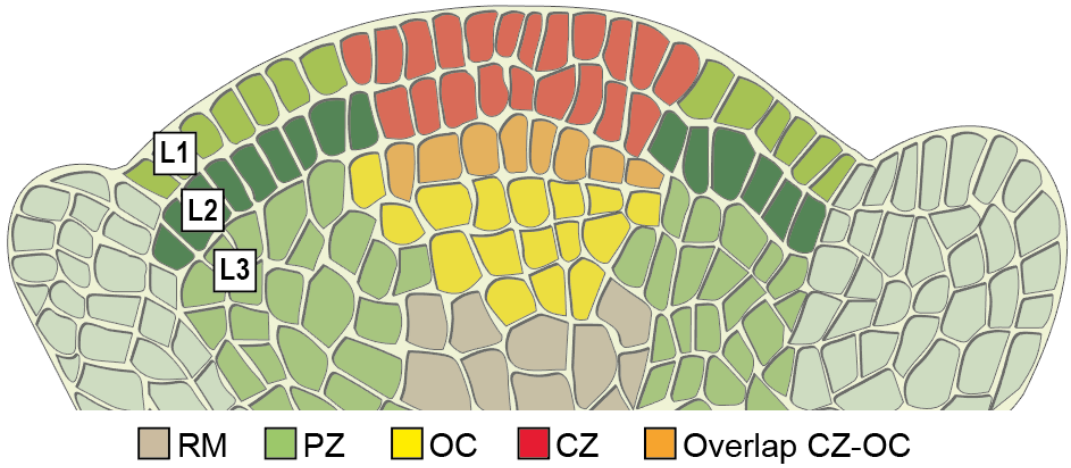

Figure 1| Arabidopsis shoot apical meristem organization
RM: Rib meristem, PZ: Peripheral zone, OC: Organizing centre, CZ: central zone. L1 to L3: Layer 1 to layer 3. Adapted from *Lopes et al., 2021*.

1.2. Cytokinin

Cytokinins are key regulators of numerous growth and developmental processes in plants, including root and shoot morphogenesis and maintenance, cell division and leaf senescence (Nguyen et al., 2021). CKs are also essential for responses to abiotic and biotic factors (Cortleven et al., 2019).

CK biosynthesis is mediated by the activity of the adenosine phosphate-isopentenyl transferases (IPTs) that produce riboside precursors. The precursors are then transformed into active CKs by enzymes from the

LONELY-GUY (LOG) family (Kieber and Schaller, 2014). The pattern of expression of IPT and LOG enzymes support that CKs can serve as autocrine signals, acting locally at the site of synthesis, but that they can also act systemically, travelling through the vasculature of the plant (Kieber and Schaller, 2014).

CKs are perceived by the ARABIDOPSIS HISTIDINE KINASE 2–4 (AHK2–4) receptors which initiate a phosphorylation-based signalling cascade that ultimately leads to phosphorylation and activation of B-type ARABIDOPSIS RESPONSE REGULATORS (ARRs) by the ARABIDOPSIS HISTIDINE PROTEINS (AHPs). In turn, active ARRs induce the expression of CK-responsive genes, such as those encoding CYTOKININ RESPONSE FACTORS (CRFs) and A-type ARRs, repressors of CK signalling that help to reset the pathway (Kieber and Schaller, 2014).

There are two major types of CKs: the N6-isopentenyl-adenine type (iP) that mostly acts in the root, and the trans-zeatin type (tZ) that is essential for shoot function and is the most abundant and active CK form in all plant species (Kieber and Schaller, 2014). Interestingly, the iP-type of CKs can be converted into a tZ-type by oxidation through the action of enzymes from the cytochrome P450 family (CYP735A1 and 2) that are expressed predominantly in roots but are necessary for proper shoot development (Kiba et al., 2013).

CKs can induce shoot formation from undifferentiated callus cultures or even induce the initiation of ectopic meristems, suggesting that CKs can specify shoot cell fate starting from different cell types, and that they play a role in SAM development (Kieber and Schaller, 2014). Accordingly, CK deficient mutants display reduced SAM activity (Bartrina et al., 2011). Furthermore, reduced CK levels or signalling result in a smaller SAM, suggesting that CKs are positive regulators of stem cell proliferation (Werner et al., 2003). Indeed, CKs are known to promote *WUS* expression through

direct binding of B-type ARRs to the *WUS* promoter and by repressing *CLV1* expression (Buechel et al., 2010; Gordon et al., 2009; Nimchuk et al., 2015). In turn, WUS promotes CK signalling in the OC by directly inhibiting the expression of type-A ARR repressors. Hence, WUS indirectly promotes its own expression, and thus, stem cell proliferation through CKs (Gordon et al., 2009; Leibfried et al., 2005).

The SAM also responds to changes in nitrate availability in a CK-dependent manner. Increasing nitrate levels in the soil lead to the production of CK precursors in the root that are then transported to the shoot (Osugi et al., 2017). In the SAM, LOG enzymes generate the active form of these precursors, inducing CK signalling and thus *WUS* expression (Landrein et al., 2018).

Besides WUS, KNOX transcription factors such as STM, also influence CK production by inducing the expression of *IPT7* (Scofield et al., 2013). in turn, higher CK levels promote the expression of *KNOX* genes in Arabidopsis (Rupp et al., 1999), suggesting that there is a positive feedback loop between CK levels and *KNOX* gene expression in the SAM.

1.3. Auxin

Auxin is a known regulator of numerous plant developmental responses that rely on the spatiotemporal control of cell division, growth, and differentiation (Gallei et al., 2020; Zhao, 2018).

In plants there are three naturally occurring auxins: indole-3-acetic acid (IAA), phenylacetic acid (PAA) and 4-chloro-indole-3-acetic acid (4-Cl-IAA), with IAA being the most studied and widespread across different plant species (Casanova-Sáez et al., 2021).

Contrasting with CK, which induces cell proliferation, auxin signalling promotes differentiation, guiding the formation of new lateral organs, such as leaves and flowers (Reinhardt, 2000). In the SAM, the polar transport of auxin, mediated by PIN-FORMED 1 (PIN1), leads to the generation of auxin peaks at the periphery of the SAM, that mark the position of new organs, while auxin depletion around these organs generates zones that are inhibitory for organ development (Reinhardt, 2000). Such arrangement of lateral organs along the main plant axis driven by auxin efflux and concentration gradients is generally known as phyllotaxis (de Reuille et al., 2006; Jönsson et al., 2006; Traas, 2013).

A balance between organ formation and stem cell proliferation is partly achieved through an interplay between auxin and CK signalling. In the developing organs, the transcription factor AUXIN RESPONSIVE FACTOR 5 (ARF5, also called MONOPTEROS, MP), promotes the expression of AHP6, a pseudo AHP that acts as a negative regulator of CK signalling (Mähönen et al., 2006). This mechanism restricts the activity of the CK pathway to the centre of the SAM (Besnard et al., 2014), leading to higher levels of CK in the primordia and aberrant timing of lateral organ growth in *ahp6* loss-of-function mutants (Besnard et al., 2014) (see next section on Phyllotaxis).

Besides its role in promoting cell differentiation, auxin also contributes to the maintenance of stem cell activity in the SAM, promoting WUS expression in two ways. On one hand, ARF5/MP promotes CK signalling by directly repressing the expression of *ARR7* and *ARR15*, which encode for type-A ARR CK signalling repressors (Leibfried et al., 2005; Zhao et al., 2010). This strengthens CK signalling in the meristem, promoting *WUS* expression and thus, the regulation of stem cell activity and SAM development (Zhao et al., 2010). In turn, WUS can regulate auxin signalling by triggering histone deacetylation of genes, such as *ARF5* or *IAA9*, known to be important for the

nuclear auxin pathway (NAP) and of targets acting downstream of auxin signalling (Ma et al., 2019). Thus, WUS may suppress auxin signalling through histone deacetylation of auxin signalling genes, restricting auxin signalling in the stem cell population, while at the same time allowing low levels of signalling output (Ma et al., 2019). Such changes in the chromatin state could at least partly explain the observed temporal integration of auxin signalling that is crucial for organ initiation in the SAM (Galvan-Ampudia et al., 2020). The meristem generates spatio-temporal auxin gradients derived from rhythmic auxin fluxes formed as protrusions originated from the CZ (Galvan-Ampudia et al., 2020). Hence, the progressive exposure of cells exiting the CZ to these auxin gradients could lead to progressive changes in histone acetylation that ultimately lead to differentiation and organ outgrowth.

On the other hand, ARF5/MP was also shown to repress the expression of the DORNROSCHEN/ENHANCER OF SHOOT REGENERATION 1 (DRN/ESR1) TF, a positive regulator of *CLV3* expression (Luo et al., 2018). Hence, by repressing *CLV3*, ARF5/MP contributes in a more direct manner to WUS expression and stem cell maintenance.

1.4. Phyllotaxis

As previously referred, the balance between auxin and CK is crucial not only for stem cell regulation and organogenesis but also for the precise spatiotemporal arrangement of lateral organs (leaves, branches, flowers, etc.) along the stem, commonly known as phyllotaxis (from ancient Greek: *phýllon* “leaf” and *táxis* “arrangement”). In nature, it is possible to find distinct phyllotactic patterns, such as whorled, spiral, alternate (distichous) and opposite (decussate), spiral phyllotaxis being the most common one (Jean

and Barabé, 1998). This pattern is characterized by the presence of the “golden angle”, defined from the Fibonacci series as an average divergence angle between successive organs which is close to 137.5° (Adler et al., 1997).

In plants, these characteristic developmental patterns are established by the relative time between organ initiations (called the plastochron), and a tight control of the spatial positioning of new organs (Barabé and Lacroix, 2022). This is established through a spatio-temporal control of cell activity which enables the continuous initiation of new organ primordia at precise positions while maintaining the integrity of the stem cell niche. A variety of models have been proposed to explain these patterns, but the inhibitory field hypothesis is now the most widely accepted. This model proposes that organ primordia generate inhibitory fields that prevent the formation of new organs in their vicinity (Moulia et al., 2022; Shi and Vernoux, 2019). Hence, as the SAM grows, new primordia can only develop at sites where the inhibition is lowest, thus at a certain distance from existing primordia.

As previously mentioned, auxin plays major roles in organogenesis and thus, in the determination of phyllotactic patterns. Contrasting with other phytohormones, auxin transport is mainly directional through the action of carriers such as PIN1, which is necessary for organ initiation. Indeed, the inflorescence meristems of *pin1* mutant mostly fail to generate lateral organs (Galweiler et al., 1998; Okada et al., 1991). This phenotype can however be rescued by applying locally a high concentration of auxin (Reinhardt, 2000), demonstrating that auxin efflux is essential for organogenesis in the inflorescence meristem and that the generation of localized peaks of auxin concentration are necessary and sufficient for the formation of new organ primordia in the SAM.

Auxin influx carriers also play an essential role in auxin distribution dynamics, and thus are also involved in the regulation of phyllotaxis by this

phytohormone. Mutants of the AUXIN1/LIKEAUXIN1 (AUX/LAX) family of auxin influx carriers show clear phyllotaxis alterations, showing that cellular auxin influx is also required to stabilize phyllotactic patterns in the SAM (Bainbridge et al., 2008). This supports the hypothesis that polar auxin transport triggers organ initiation and at the same time establishes inhibitory fields through depletion of the hormone in the surrounding regions (Douady and Couder, 1996; Moulia et al., 2022; Shi and Vernoux, 2019).

CK is also required for organogenesis along with auxin (Yoshida, Mandel, & Kuhlemeier, 2011). In the SAM, the spatio-temporal distribution of the CK signalling repressor AHP6, which is dictated by ARF5/MP-mediated auxin signalling (Besnard et al., 2014), was shown to be necessary for optimizing the rhythmicity of organ initiation. Low levels of AHP6 at the first primordium initiation site (i1) allow high CK signalling and organ initiation, while high levels of AHP6 at the second initiation site (i2) have the contrary effect, preventing organ co-initiation and stabilizing the plastochron (Besnard et al., 2014). Thus, in addition to the primary auxin inhibitory fields that specify primordia initiation, AHP6 acts downstream of auxin to produce a secondary CK-dependent inhibitory field that increases the robustness of the timing of the phyllotaxis pattern (Besnard et al., 2014). In accordance with such model, *ahp6* meristems produce up to 3 organs simultaneously, rather than sequentially as wild-type meristems. These organs are nevertheless produced at their expected position with no changes in the divergence angle between organ initiation sites (Besnard et al., 2014).

Besides hormones, mounting evidence implicates mechanical signals in SAM patterning and phyllotaxis. Changes in cell wall rheology are likely to influence cell growth rate and anisotropy (Hamant and Traas, 2010), both of which are essential for organogenesis and thus phyllotaxis (Cosgrove, 2005; Sablowski and Carnier Dornelas, 2014). Indeed, the SAM shows locally

distinct mechanical properties and the increased cell wall elasticity of growing primordia, as compared to other regions of the SAM, supports a correlation between changes in mechanical properties and organogenesis (Kierzkowski et al., 2012; Milani et al., 2011; Peaucelle et al., 2011; Robinson et al., 2013).

Moreover, during the formation of organ primordia, auxin signalling can induce cell wall acidification and the activation of cell wall remodelling enzymes, altogether leading to irreversible cell wall expansion (i.e. growth) (Arsuffi and Braybrook, 2018; Murray et al., 2012; Peaucelle et al., 2011). Cell mechanical properties in the SAM are also controlled by microtubules, which guide cellulose microfibril deposition, influencing cell wall anisotropy and thereby the direction of cell growth (Bringmann et al., 2012; S. Li et al., 2012; Shi and Vernoux, 2019). Accordingly, induction of organ outgrowth by auxin also involves a depolymerization of the microtubules (Sassi et al., 2014). Growth and shape-driven mechanical forces can also affect microtubule organization (Hamant et al., 2008), influencing cell growth anisotropy and thus organ emergence in the SAM (Uyttewaal et al., 2012).

In addition to its impact on the cytoskeleton, it has also been suggested that mechanical forces could also affect PIN1 polarity, and thus auxin distribution in the SAM, which could thereby influence phyllotaxis (Heisler et al., 2010; Nakayama et al., 2012, Landrein et al, 2015).

In summary, since very early in development, different chemical and physical signals converge into the SAM to define the phyllotactic pattern established through a tight spatio-temporal control of new primordium formation. This self-organization seems to follow the inhibitory field hypothesis, where existing organs block the initiation of new primordia in their vicinity through the regulation of key players such as auxin and CK.

1.5. Homeodomain transcription factors and the control of SAM development

Since the Antiquity, botanists noticed the presence of flowers where an entire organ was replaced by another one (Hamant and Pautot, 2010). These phenotypes are now known to be caused by mutations in homeotic genes, encoding transcription factors that harbour an evolutionarily conserved helix-turn-helix DNA binding domain, known as the homeobox or homeodomain (HD). Homeobox transcription factors are known regulators of morphological pattern formation in both animals and plants, playing important roles in a variety of processes, including the establishment of cell and tissue identity, and the regulation of cell proliferation, differentiation, and survival (Hamant and Pautot, 2010; Maksimova et al., 2021).

Homeobox transcription factors generally carry a 60 amino acid DNA-binding homeodomain that folds into three α-helices and that is responsible for DNA binding. The TALE (three-amino acid length extension) superclass of homeobox transcription factors is characterized by a three-amino-acid insertion of a proline – tyrosine – proline, between α-helix 1 and 2 (Burglin, 1997).

In animals, TALE proteins can be divided into two subfamilies: the PBC family, which includes the vertebrate Pbx proteins, and the MEIS family, that comprises the vertebrate Meis and Prep proteins. Both PBC and MEIS proteins contain a TALE motif and are known to form heterodimers with the HOX-class homeobox transcription factors, which are known regulators of the formation of the anterior-posterior axis during embryogenesis (Mann et al., 2009).

In plants, KNOX (KNOTTED1-like homeobox) proteins share homology with the animal MEIS in their heterodimerization domain, which led to the

proposal that they evolved from a common ancestral TALE class named MEI-NOX (Burglin, 1997). Besides KNOX proteins, plants have a second group of TALE homeobox proteins referred to as the BELL (BEL1-like) class (Burglin, 1997). Like for the PBC-MEIS proteins, the interaction between KNOX- and BELL-class transcription factors in the TALE group also plays critical roles during plant development, such as the regulation of SAM formation and maintenance, organ morphogenesis and position, and several other aspects of the reproductive phase (Hake et al., 2004; Hamant and Pautot, 2010) (for a more detailed description, see below).

The DNA-binding specificity of TALE proteins mainly depends on the homeodomain residues at positions 47, 50, and 54 which differs between TALE members. By forming heterodimers, TALE proteins increase the target affinity by bringing two DNA-binding domains together (Lee et al., 2008; Noyes et al., 2008; Smith and Hake, 2003).

In animals, PBX proteins are localized in the cytosol and, upon heterodimerization with MEIS proteins, they are imported to the nucleus (Berthelsen et al., 1999; Stevens and Mann, 2007). In plants, this heterodimerization-dependent translocation is also observed in the KNOX-BELL pair, suggesting that this mechanism is conserved between TALE proteins (Bhatt et al., 2004).

In Arabidopsis, the TALE homeodomain superclass of transcription factors comprises 9 KNOTTED-like (KNAT or KNOX) and 13 BEL1-like (BLH or BELL) members. The KNOX family is divided into three classes: class I includes STM, BREVIPEDICELLUS (BP)/KNAT1, KNAT2 and KNAT6; class II includes KNAT3, 4, 5, and 7; class III contains KNATM, which produces three isoforms by alternative splicing and has no homeodomain (Figure 2) (reviewed in Hamant and Pautot, 2010).

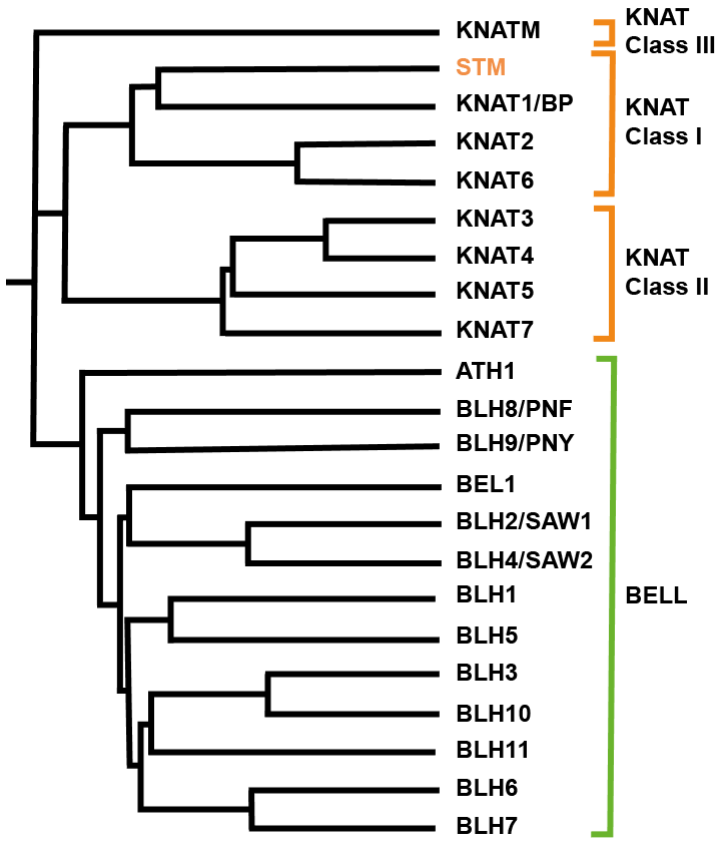

Figure 2 | Phylogeny of the TALE superfamily (adapted from Hamant and Pautot, 2010).

KNOX members are generally distinguished by four characteristic domains: the N-terminal KNOX1 and KNOX2 domains (collectively known also as the MEINOX domain), the ELK domain, and the C-terminal HD (Figure 3) (Bürglin, 1998; Burglin, 1997; Vollbrecht et al., 1991). The MEINOX domain is known to mediate the interaction between KNOX proteins, such as STM, and BELL transcription factors, thereby promoting the translocation of KNOX to the nucleus and thus, the expression of KNOX target genes (Bellaoui et al.,

2001; Bhatt et al., 2004; Cole et al., 2006; Smith and Hake, 2003). The HD plays a crucial role in DNA binding but also in intercellular trafficking (Cole et al., 2006; Smith et al., 2002; Viola and Gonzalez, 2009). The ELK domain harbours a highly conserved S residue (S272) whose phosphorylation was shown to be involved in the nucleo-cytoplasmic distribution of STM (Aguilar-Martínez et al., 2015). Mutation of this residue to prevent phosphorylation (S272A) leads to constitutive localization of STM to the nucleus, suggesting that S272 phosphorylation modulates the interaction of STM with its partner proteins and hence its nuclear localization (Aguilar-Martínez et al., 2015). A relatively small and less well-conserved motif, known as the GSE domain, is located between the MEINOX and the ELK domains. This motif harbours a PEST sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T) that is involved in the ubiquitin-mediated degradation of proteins. Nevertheless, the role suggested for the GSE domain in KNOX protein degradation has not yet been fully unveiled (Nagasaki et al., 2001; Vollbrecht et al., 1991).

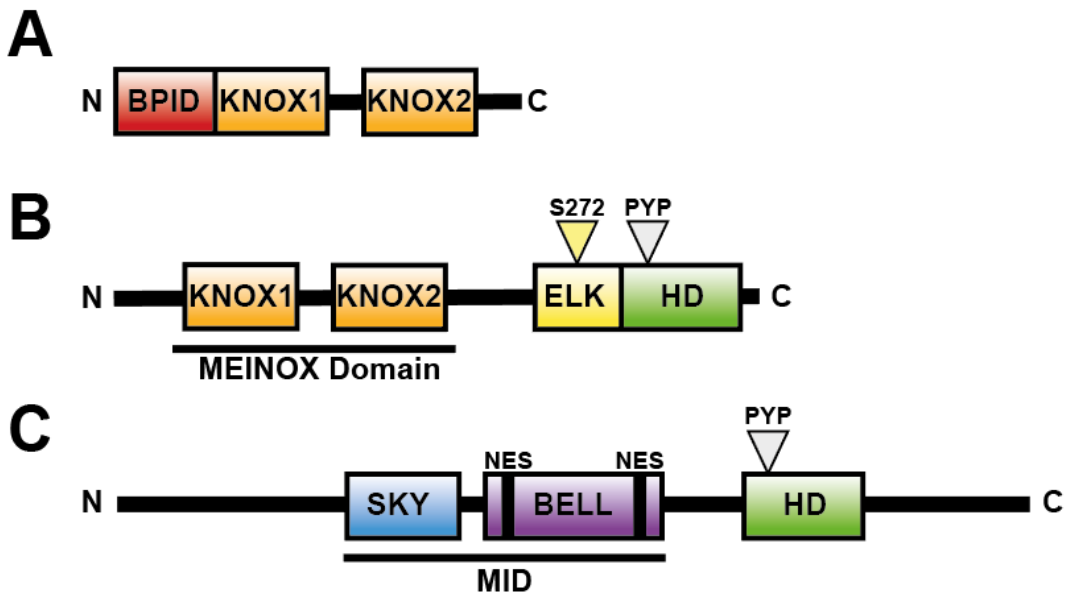

Figure 3 | Schematic structures of the KNOX and BELL proteins.
Schematic structure of the KNAT class III (A), KNAT class I and II (B) and BELL proteins (C). KNAT proteins contain a MEINOX domain (harbouring two subdomains, KNOX1 and KNOX2) which is responsible for the interactions with other KNOX and TALE proteins. Class I and II of the KNAT family are also characterized by an ELK domain, that harbours a highly conserved S residue (S272), and a TALE homeodomain (HD) which has a three-amino-acid insertion of a proline – tyrosine – proline (PYP), between α-helix 1 and 2. The KNATM protein (A) has no HD and interacts through the BPID (BP interacting domain). BELL proteins contain a TALE HD, a MID (MEINOX interacting domain) which comprises the SKY and BELL regions. The BELL domain contains two nuclear exclusion leucine-rich sequences (NES) that are also important for the interaction with STM.

STM loss-of-function, in strong alleles such as *stm-1*, leads to the loss of a functional SAM, showing growth arrest at the seedling stage and the fusion of the cotyledonary petioles. In contrast, weaker *stm* mutants, do form a SAM that initiates primordia shortly after germination, but the stem cell niche is eventually depleted as a result of the differentiation and incorporation of stem cells in organ primordia. However, growth may occur from adventitious meristems that form on the petiole of the cotyledon in some of these weaker alleles. These adventitious meristems are defective and unable to support growth, thus resulting in the formation of extra adventitious or axillary shoots. Although weak *stm* mutants do form flowers, they normally lack some of the reproductive organs (such as the gynoecium) and often show a reduced number of fused stamen and petals. Reduced STM expression in knock-down mutants leads also to clustered internodes and sepal fusion defects (Barton and Poethig, 1994; Clark et al., 1996; Endrizzi, Karin K. et al., 1996; Long et al., 1996; Song et al., 2020)

Heterodimerization between KNOX and BELLs has been shown to be essential for the nuclear localization of KNOX proteins, as well as for their binding affinity to DNA and thus, their function (Cole et al., 2006; Kim et al., 2013; Rutjens et al., 2009; Smith et al., 2002; Viola and Gonzalez, 2009). Interestingly, the conserved BELL domain is sufficient for the interaction with STM, but not for the nuclear import of the STM protein (Cole et al., 2006), analogous to what has been reported for PBC/MEIS interactions (Cole et al., 2006; Kilstrup-Nielsen et al., 2003).

The BELL family in Arabidopsis comprises BLH9 [also known as PENNYWISE (PNY), BELLRINGER, REPLUMLESS, LARSON or VAAMANA], POUNDFOOLISH (PNF)/BLH8, A. THALIANA HOMEOBOX GENE1 (ATH1), SAWTOOTH1 (SAW1)/BLH2, SAWTOOTH2 (SAW2)/BLH4, and BEL1 – whose functions have been better characterized - and BLH1, BLH3, BLH5, BLH6, BLH7, BLH10, and BLH11 – whose functions are not yet fully understood (Hamant and Pautot, 2010).

The BELL domain harbours two leucine-rich nuclear export signals (NES), that are known to interact with the nuclear export receptor CRM1/exportin-1. These sequences are also sufficient for the interaction with STM, suggesting that STM may compete with the nuclear export machinery for binding to the BELL domain and in that way promote the retention of the BELL proteins in the nucleus (Rutjens et al., 2009).

PNY is one of the best studied members of the BELL family. Loss-of-function *pny* mutants show loss of the replum, inflorescence patterning defects and, enhancement of weak *stm* phenotypes (Bhatt et al., 2004; Byrne et al., 2003; Kanrar et al., 2008, 2006; Roeder et al., 2003; Smith and Hake, 2003). PNY also plays a role in defining the spatial expression patterns of boundary genes, which directly impact flowering regulation by FLOWERING LOCUS T (FT), the mobile factor that promotes floral induction in response to day length

(Andrés et al., 2015). Additionally, by repressing organ boundary genes, PNY also regulates the rib zone function by establishing the central and peripheral regions (Bencivenga et al., 2016).

Besides its genetic interaction with STM, PNY also interacts with BP, with *pny* mutations synergistically enhancing the inflorescence phenotype (e.g. reduced height or irregularly shortened internodes) of the *bp* mutant (Bhatt et al., 2004; Byrne et al., 2002; Smith and Hake, 2003; Venglat et al., 2002). The paralogous gene of PNY, PNF/BLH8, was also shown to heterodimerize with STM. However, unlike *pny*, single *pnf* loss-of-function alleles do not enhance *stm* phenotypes (Kanrar et al., 2008; Smith et al., 2004) and play a role in inflorescence development only when combined with PNY. Both PNY and PNF are required for proper STM function; however, the phenotype of *pny pnf* is distinguishable from that of *stm*, suggesting that PNY and PNF are involved in developmental processes that are not always shared with STM (Ung et al., 2011).

A physical interaction with STM has also been reported for ATH1, which also interacts with BP/KNAT1, KNAT2 and KNAT6 (Cole et al., 2006; Hackbusch et al., 2005; Y. Li et al., 2012). The *ATH1* gene was first identified as a target of the photomorphogenic proteins CONSTITUTIVE PHOTOMORPHOGENIC1 (COP1) and DEETIOLATED1 (DET1), as *ATH1* was derepressed in *cop1* and *det1* mutants (Quaedvlieg et al., 1995). Indeed, *ATH1* and light-activated genes play redundant functions in the inhibition of cell proliferation to prevent stem growth during the vegetative phase. In turn, *ATH1* is downregulated when stem growth is activated during the flowering stage (Gomez-Mena and Sablowski, 2008). ATH1 was shown to play a role also in the establishment of boundaries between the shoot organs and the stem, in the regulation of SAM activity, and in repressing flowering *via*

activation of FLOWERING LOCUS C (FLC) (Cole et al., 2006; Gomez-Mena and Sablowski, 2008; Proveniers et al., 2007; Rutjens et al., 2009).

1.6. The role of energy signalling in development

The proper development of an organism requires a tight regulation between cell proliferation and differentiation into tissues and organs. The decision to promote or restrain cell proliferation and growth relies in the integration of external and internal cues with information on the available energy resources. Energy levels are sensed by dedicated mechanisms that in turn modify physiology and growth with the ultimate aim of restoring energy levels to an optimum (Garcia and Shaw, 2017; Mohammed et al., 2017; Polge and Thomas, 2007; Wu et al., 2019).

One major player in eukaryotic energy sensing and signalling is the highly conserved protein kinase complex TARGET OF RAPAMYCIN (TOR). TOR is down-regulated under stress conditions that restrict sugar availability and is globally activated in response to nutrient availability, promoting cell proliferation, growth, and developmental progression (Liu and Xiong, 2021). Accordingly, in the SAM of plants grown under darkness and without sugar supplementation, TOR activity is inhibited, and cell proliferation is halted. Glucose feeding can rescue TOR activity and growth in the RAM, but in the SAM, such rescue requires both glucose and light, with the effect of the latter being auxin-mediated (X. Li et al., 2017). TOR promotes SAM activity by upregulating the expression of S-phase genes *via* phosphorylation of the E2Fa transcription factor (Li et al., 2017; Xiong et al., 2013). In addition, TOR promotes *WUS* expression at least partly by inhibiting CK degradation (Janocha et al., 2021; Pfeiffer et al., 2016).

In all eukaryotes, TOR operates in a largely antagonistic manner to another highly conserved protein kinase that belongs to the calcium-dependent protein kinase (CDPK) family: Sucrose Non-Fermenting 1 (SNF1) in yeast, AMP-activated kinase (AMPK) in mammals, and SNF1-related kinase 1 (SnRK1) in plants (González et al., 2020; Jamsheer K et al., 2021). SNF1/AMPK/SnRK1 kinases are activated when energy levels decline, inducing an energy-saving program through the coordinated upregulation of catabolic, energy-producing pathways and downregulation of anabolic, energy-consuming processes.

In yeast cells, SNF1 is fundamental for the adaptation to low glucose conditions, leading to a drastic metabolic shift that allows the utilization of less preferred carbon sources that cannot be fermented, such as sucrose (from which the Sucrose Non-Fermenting 1 name derives), galactose, and ethanol (Hedbacker, 2008).

In animals, AMPK controls energy homeostasis both at the cellular and whole-organism levels, contributing to glucose homeostasis through the regulation of processes such as insulin production and glucose uptake, lipid breakdown and appetite (Hardie et al., 2012). Mounting evidence also implicates AMPK in cell fate decisions and various developmental processes (Dasgupta and Milbrandt, 2009; Ramamurthy et al., 2014; Rashid et al., 2021; Sarikhani et al., 2020; Young et al., 2016).

Similarly to SNF1 and AMPK, SnRK1 is activated under low carbon conditions in plants to promote energy-saving and nutrient remobilization strategies through a vast transcriptional and metabolic reprogramming (Figure 4) (Jamsheer K et al., 2021). Plants experience low carbon conditions when exposed *e.g*. to drought, flooding, extreme temperatures, high salinity, or attacks from various pathogens, as most stresses interfere with photosynthesis and/or respiration and ultimately result in impaired energy

production (Baena-González et al., 2008; Hulsmans et al., 2016; Margalha et al., 2019). Accordingly, plants with constitutive activation of the SnRK1 pathway are more resistant to a wide variety of stresses whilst the opposite is also true for plants with reduced SnRK1 activity (Broeckx et al., 2016; Margalha et al., 2019). In addition, and like for AMPK, SnRK1 has been implicated in virtually all aspects of plant development (Baena-González and Hanson, 2017; Jamsheer K et al., 2021), suggesting that it is involved in the integration of environmental information into growth and developmental decisions.

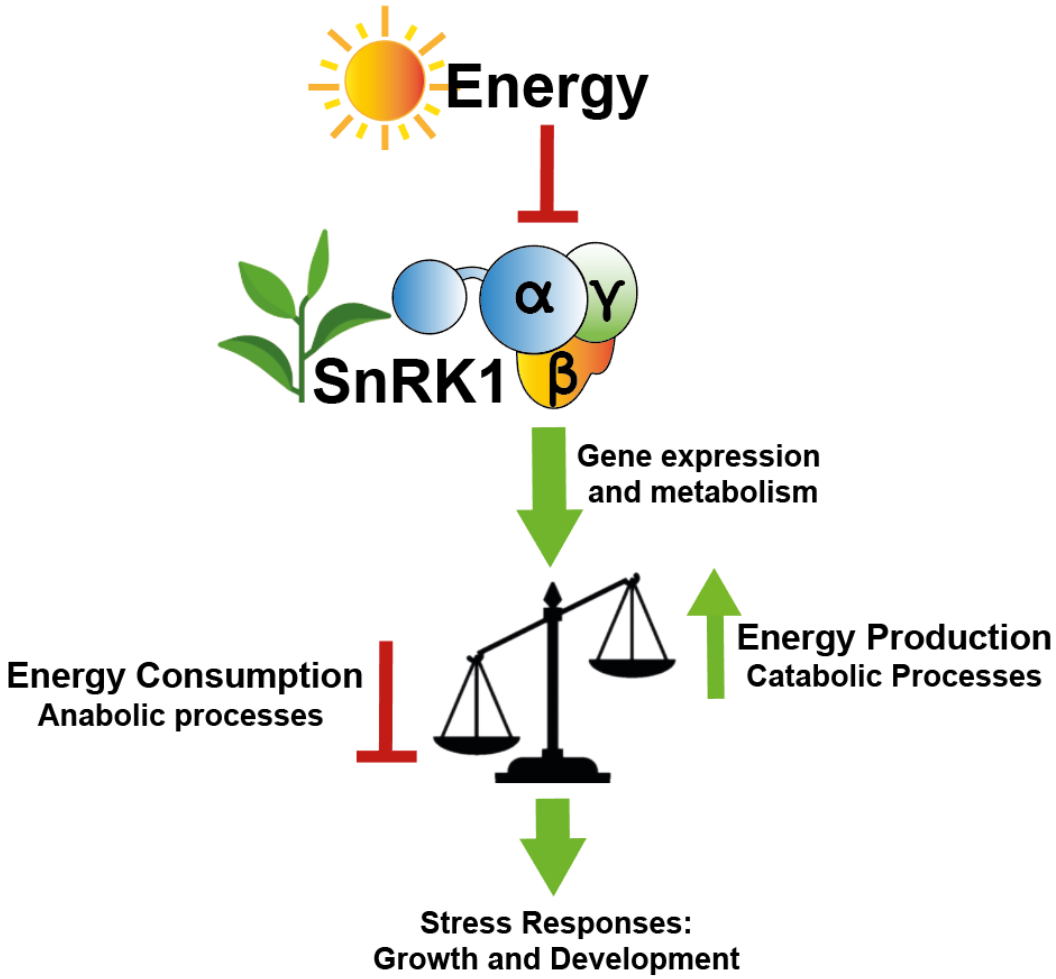

Figure 4 | Regulation of SnRK1 activity

When energy levels are low, SnRK1 is activated and exerts its function by transcriptional reprogramming and direct phosphorylation of metabolic enzymes and regulatory proteins. In this way, SnRK1 can effectively implement an energy-saving programme, repressing anabolic processes and promoting catabolism, ultimately impacting on metabolism, stress responses, growth, and development.

1.7. Sucrose Non-Fermenting-related kinase 1 (SnRK1)

The SnRK super family comprises three distinct subfamilies in plants, SnRK1, SnRK2, and SnRK3. In Arabidopsis, the SnRK2 and SnRK3 subfamilies have 10 and 25 members, respectively, and are mostly known for their role in stress and abscisic acid (ABA) signalling (Halford and Hey, 2009; Umezawa et al., 2010).

SnRK1 is a heterotrimeric protein complex, composed of a catalytic α-subunit and two regulatory subunits, β and βγ (Figure 5) (Jamsheer K et al., 2021). In Arabidopsis, the α-subunit is encoded by 3 genes: *SnRK1α1*, *SnRK1α2* and *SnRK1α3* (also known as *SnRK1.1/SnRK1.2/SnRK1.3*, *AKINα1/AKINα2/AKINα3*, *KIN10/KIN11/KIN12*, or *AKIN10/AKIN11/AKIN12*). Additionally, three genes encode for the β subunit (*SnRK1β1/β2/β3*), and one for a SnRK1βγ hybrid subunit (Jamsheer K et al., 2021).

The catalytic α-subunit is characterized by an N-terminal kinase domain, and a C-terminal regulatory domain which is necessary for the formation of the complex through the interaction with the β- and βγ-subunits (Broeckx et al., 2016). The N-terminal catalytic domain is highly conserved between

eukaryotes and harbours a conserved threonine residue (T175 for AtSnRK1α1, and T176 for AtSnRK1α2) in the kinase activation loop (T-loop), whose phosphorylation is essential for kinase activity (Baena-González et al., 2007; Estruch et al., 1992; Hawley et al., 1996).

The regulatory β-subunits perform a scaffolding function and are thought to play a role in the subcellular localisation of the complex, in substrate recognition, and in the general kinase activity (Hardie et al., 2012; Hedbacker et al., 2004; Jamsheer K et al., 2021; Polge and Thomas, 2007). SnRK1β1 and SnRK1β2 have a conserved N-terminal domain that is myristoylated *in planta*, resulting in the anchoring of this protein to membranes (Pierre et al., 2007; Wang et al., 2020), a carbohydrate binding module (CBM), and a β C-terminal domain (βCTD) that is key for binding to the α- and the βγ-subunits (Broeckx et al., 2016).

Finally, SnRK1βγ is characterized by four cystathionine β-synthase (CBS) domains organized in tandem pairs (also known as the Bateman domains), and by the β-interacting sequence domains, both typical of the γ-subunit of SNF1/AMPK/SnRK1 kinases. However, in plants this subunit also has features characteristic of the β subunits, such as the CBM domain (Jamsheer K et al., 2021). The Arabidopsis *snrk1βγ* mutant is not viable, indicating that this subunit plays unique and essential functions (Ramon et al., 2013). SnRK1βγ was shown to be important for pollen hydration and germination (Gao et al., 2016), suggesting that the inability to retrieve a homozygous *snrk1βγ* mutant could at least partly be explained by defective pollen transmission.

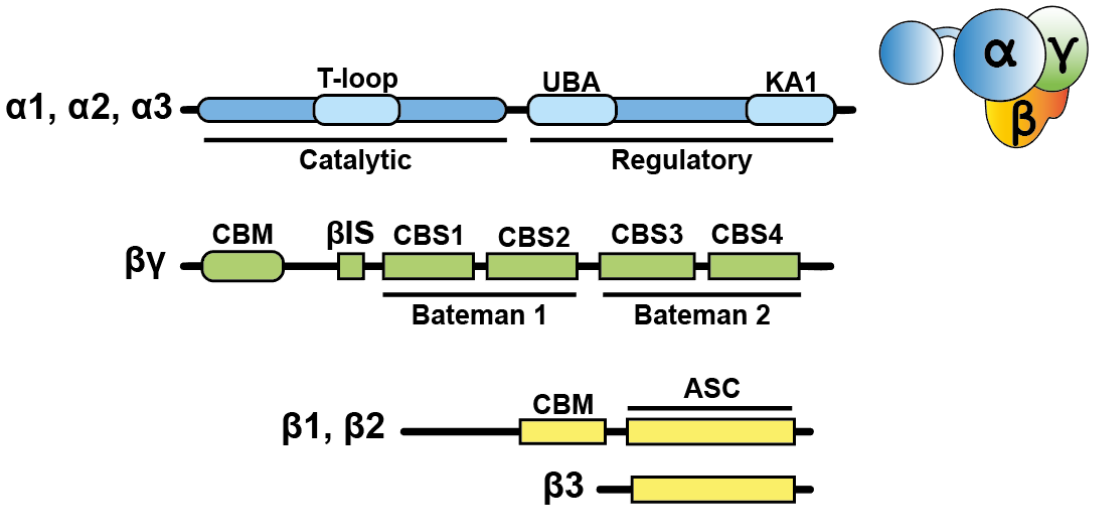

Figure 5 | SnRK1 is a heterotrimeric complex.

The α-subunit (in blue) is composed of a catalytic domain harbouring the conserved T-loop, and a regulatory domain, where we can find a ubiquitin-associated (UBA) domain, and a kinase-associated 1 (KA1) domain which are essential for the interaction between SnRK1α with the regulatory SnRK1β and SnRK1γ subunits and with other regulatory proteins. The SnRK1βγ (in green) has two Bateman domains, each containing two cystathionine-β-synthase (CBS) motifs, and a β-interacting sequence (βIS). SnRK1βγ unconventionally harbours a carbohydrate-binding module (CBM). The N-terminal region harbours a carbohydrate binding module (CBM), separated from the first Bateman domain by the βIS. The β-subunit (in yellow), harbour a Kinase Interacting Sequence (KIS) and the Association to the Snf1 Complex (ASC) domain, containing sites of interaction with α and γ. The SnRK1β3 is an atypical β-subunit, as it does not preserve the N-terminal variable extension or the CBM due to an N-terminal truncation.

SnRK1α1 is expressed early on during embryogenesis and throughout several developmental stages, and is also ubiquitously expressed in different tissues, being most enriched in leaf primordia, seedling root tips, and

vasculature. On the other hand, *SnRK1α2* expression is more restricted in both space and time, being mostly found at the hydathodes, and the newly developing leaves (Jossier et al., 2009; O'Brien et al., 2015; Williams et al., 2014). As for *SnRK1α3*, its expression was only weakly detected in pollen grains, developing embryos and seeds (Baena-González et al., 2007; Fragoso et al., 2009; Schmid et al., 2005). The SnRK1α1 and SnRK1βγ proteins are also enriched in the meristematic, elongation and differentiation zones of both primary and lateral roots, as well as in young leaf primordia (Belda-Palazón et al., 2020; Bitrián et al., 2011).

All three β-subunits are expressed in roots and mature rosettes, whereas in reproductive tissues, such as the developing pollen grain, the ovule and the seed, *SnRK1β3* appears to be the predominant one (Polge and Thomas, 2007).

Post-translational modifications are essential for the regulation of SnRK1 kinase activity (Broeckx et al., 2016; Jamsheer K et al., 2021). Similarly, to AMPK and SNF1, SnRK1α activity requires phosphorylation of the highly conserved T-loop threonine (Baena-González et al., 2007; Sugden et al., 1999) by the SnRK1-Activating Kinases (SnAKs) (Hey et al., 2007; Shen et al., 2009). SnAK1 and SnAK2, also known as GRIK2 and GRIK1 respectively, interact with the SnRK1 catalytic subunits, phosphorylating the T-loop in a Ca^{2+}-independent manner and leading to SnRK1 activation (Shen et al., 2009). In animals, AMPK activation is commonly accompanied by an increase in T-loop phosphorylation; hence T-loop phosphorylation is widely used as readout of AMPK activity (Lin and Hardie, 2018). In contrast, a clear correlation between SnRK1 activity and T-loop phosphorylation has only been reported in plants exposed to flooding, with low oxygen under these conditions leading to a marked increase in T-loop phosphorylation (Cho et al., 2016). However, how SnRK1 activation occurs is still not fully understood, since such

correlation between T-loop phosphorylation and SnRK1 activity has not been observed in other stress conditions (Baena-González et al., 2007; Fragoso et al., 2009; Rodrigues et al., 2013). SnRK1 activity was shown to be repressed by the regulatory sugar, trehalose 6-phosphate (Nunes et al., 2013; Zhang et al., 2009), which impairs the interaction between SnRK1α and SnAKs and thereby prevents SnRK1α phosphorylation and activation (Zhai et al., 2018). SnRK1α also appears to be regulated by physical obstruction, allowing it to respond to ABA (Abscisic Acid) (Belda-Palazón et al., 2020; Rodrigues et al., 2013). In the absence of ABA, the SnRK1 pathway is inactive because SnRK1α is sequestered by core components of ABA signalling, SnRK2 kinases and PP2C phosphatases. In the presence of ABA, these repressor complexes dissociate in response to ABA signalling (Cutler et al., 2010), releasing not only SnRK2s but also active SnRK1α (Belda-Palazón et al., 2020; Rodrigues et al., 2013). It is also possible that, in addition to the physical repression, containment of SnRK1α in these repressor complexes leads to partial T-loop dephosphorylation by PP2Cs (Rodrigues et al., 2013).

Other post-translational mechanisms, including ubiquitination, SUMOylation and myristoylation, have also been reported to play a role in SnRK1 regulation (Ananieva et al., 2008; Carvalho et al., 2016; Crozet et al., 2016; Jamsheer K et al., 2021). Of particular relevance for the connections between SnRK1 and development is myristoylation. Myristoylation is essential for AMPK and SNF1 regulation, mostly by enabling the association of the kinase complex to membranes and thereby affecting its subcellular localisation and interaction with target proteins (Garcia and Shaw, 2017; Hedbacker, 2008). Similarly to the animal and yeast counterparts, the SnRK1β1 and β2 subunits are also N-myristoylated, leading to the association of the complex with membranes and exclusion of the catalytic subunit from the nucleus (Pierre et al., 2007; Ramon et al., 2013). Intriguingly, N-

MYRISTOYLTRANSFERASE 1 (NMT1), a major component of the myristoylation machinery, is highly enriched in the meristems and *nmt1-1* mutants showed malformation of the SAM (Pierre et al., 2007). These defects could be traced back to the impaired myristoylation of 6 proteins, including the SnRK1β1 and β2 subunits (Pierre et al., 2007).

1.8. Thesis Outline

Energy sensing in meristematic tissues is essential to coordinate cell growth and differentiation according to the available energy resources. The functional relationship between SnRK1 and TOR serves as an important interface to integrate information on the nutrient and energy status with growth decisions at the cellular and whole-organism levels (Margalha et al., 2019). However, whether the energy status of the plant influences SAM activity and how, is still poorly understood. Therefore, this thesis aims to unveil whether different environmental and metabolic signals converge into the SAM, and to explore the underlying mechanisms, with a particular focus on the role of SnRK1 in SAM maintenance and activity.

The thesis is composed of 4 chapters (including this introductory Chapter 1) that are structured as follows:

Chapter 2:

Chapter 2 is based on a recently published review that highlights the most recent advances in our comprehension of the molecular mechanisms of WUS function, of its interaction with other transcription factors and hormonal signals, and of its connection to environmental signals.

Chapter 3:

In Chapter 3, I demonstrate that sugars promote STM accumulation and that the SnRK1 energy sensor plays a dual role in the SAM, limiting STM abundance under unfavourable conditions but being required for overall meristem organization and integrity under normal conditions.

Chapter 4:

In Chapter 4, the molecular connection between the SnRK1 kinase and STM function is explored. My results show that the SnRK1α1 catalytic subunit interacts physically with several key regulators of the SAM, such as STM and its partner transcription factors BLH8, BLH9, ATH1, and WUS, and that it strongly impinges on STM activity, potentially *via* phosphorylation.

1.9. References

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CHAPTER II

WUSCHEL in the shoot apical meristem: old player, new tricks

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2.1. Abstract

The maintenance of the stem cell niche in the shoot apical meristem, the structure that generates all of the aerial organs of the plant, relies on a canonical feedback loop between WUSCHEL (WUS) and CLAVATA3 (CLV3). WUS is a homeodomain transcription factor expressed in the organizing centre that moves to the central zone to promote stem cell fate. CLV3 is a peptide whose expression is induced by WUS in the central zone and that can move back to the organizing centre to inhibit WUS expression. Within the past 20 years since the initial formulation of the CLV–WUS feedback loop, the mechanisms of stem cell maintenance have been intensively studied and the function of WUS has been redefined. In this review, we highlight the most recent advances in our comprehension of the molecular mechanisms of WUS function, of its interaction with other transcription factors and hormonal signals, and of its connection to environmental signals. Through this, we will show how WUS can integrate both internal and external cues to adapt meristem function to the plant environment.

2.2. Introduction

Plants generate organs throughout their life thanks to the maintenance of stem cell niches localized in specialized tis-sues referred to as meristems. The shoot apical meristem (SAM) is a highly organized structure that is responsible for the generation of all of the aerial organs of the plant (Barton, 2010). Meristematic cells are characterized by the expression of SHOOTMERISTEM LESS (STM), which encodes a homeodomain transcription factor (TF) whose activity is necessary for SAM maintenance (Long et al., 1996). Stem cells are located at the centre of the SAM in the central zone (CZ) (Fig. 1). They grow and divide relatively slowly and are marked by the expression of the CLAVATA3 (CLV3) gene (Fletcher, 1999). The organizing centre (OC) is located below the CZ at the tip of the rib meristem and is defined by the expression of the stem cell regulator WUSCHEL (WUS) (Mayer et al., 1998). Cells that are advected away from the CZ through growth and division join the peripheral zone (PZ), where growth is more pronounced and where organs are initiated following specific patterns of phyllotaxis (Barton, 2010). The SAM is also organized into layers (L1, L2, and L3), based on cell lineage analysis and characteristic cell division orientations (Poethig, 1987) (Fig. 1). More than 20 years ago, the maintenance of the stem cell pool in the SAM of the model plant Arabidopsis was pro-posed to be controlled by a feedback loop between WUS and CLV3 (Brand, 2000; Schoof et al., 2000) (Fig. 1). WUS is a mobile homeodomain TF expressed in the OC that can move to the CZ to promote stem cell fate, notably by repressing differentiation (Yadav et al., 2011; Daum et al., 2014). Plants lacking WUS expression are unable to maintain their stem cell niche in the SAM, which leads to termination of the meri-stem after the production of a very limited number of organs (Laux et al., 1996). CLV3 is a small peptide whose expression is induced by WUS in the CZ but that can

repress WUS ex-pression (Brand et al., 2002). Plants lacking CLV3 expression generate very large meristems producing many organs be-cause of a lack of inhibition of WUS expression (Clark et al., 1995; Fletcher, 1999). Several CLV3 receptors have been isolated including CLV1, CLV2, CORYNE (CRN) co-receptors, members of the BARELY ANY MERISTEM (BAM) family, and the recently characterized members of the CLAVATA3 INSENSITIVE RECEPTOR KINASE (CIK) family (Clark et al., 1993; Jeong et al., 1999; DeYoung et al., 2006; Müller et al., 2008; Nimchuk et al., 2015; Hu et al., 2018). Expressed in the OC but also in different domains in the SAM, they act in concert to control WUS expression by forming a variety of homo- and heterodimers. The binding of CLV3 to CLV1 was also shown to trigger the internalization of the receptor (Nimchuk et al., 2011), a mechanism that could explain the buffering effects observed following enhancement of CLV3expression (Müller et al., 2006). As for other receptor kinases, activation of CLV1 induces a cascade of MITOGEN ACTIVATED PROTEIN KINASE activation ultimately leading to WUS repression through mechanisms that still need to be finely dissected (Betsuyaku et al., 2011). WUS also re-presses the expression of CLV1 through direct binding to its promoter, thus adding another layer of complexity to the core CLV–WUS feedback loop (Busch et al., 2010). CLV signalling also affects auxin-mediated growth in floral primordia, notably in response to cold (Jones et al., 2020). Many studies have also pushed further our comprehension of the mechanisms regulating the expression of WUS and its function in the SAM. In this review, we will discuss recent advances aiming to characterize the molecular mechanisms of WUS function, its connection to hormonal signalling, and its response to environmental cues in the model plant Arabidopsis (for a more broader view of WUS function in other species see Kitagawa and Jackson, 2019; Jha et al., 2020). WUS is also involved in floral organ identity and in floral meristem termination, but these

functions will not be discussed here (for a review on this subject, see Sun and Ito, 2015).

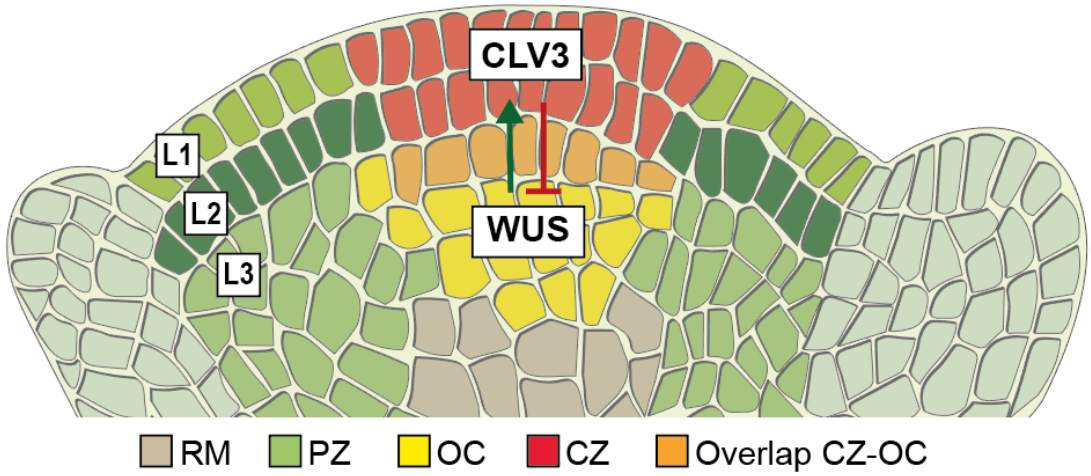


Fig.1 Organization of the Arabidopsis shoot apical meristem

RM: Rib meristem, PZ: Peripheral zone, OC: Organizing centre, CZ: central zone. L1 to L3: Layer 1 to layer 3.

2.3. Molecular mechanisms of WUS function

WUS function in the SAM relies on three distinct yet interconnected processes: its movement, its capacity to form homo- and heterodimers, and its binding specificity. WUS movement from OC to CZ is a central property of the CLV–WUS feedback loop but it was only confirmed 10 years after the formulation of the model (Schoof et al., 2000). Using a set of translational reporters fused to various florescent proteins, Yadav et al. (2011) indeed showed that WUS could move from the OC to the CZ where it directly binds to the CLV3 promoter and that this movement was necessary for WUS function. Following this work, Daum et al. (2014) showed that WUS movement

occurred through plasmodesmata and that specific sequences encoded within the WUS protein could promote but also restrict WUS movement in the SAM. It is probable that the movement of WUS through plasmodesmata is regulated by specific, yet uncharacterized proteins localized at the plasmodesmata, similarly to what has been observed for STM (Winter et al., 2007). Like other homeodomain TFs, WUS was also shown to form homodimers in vitro and in vivo (Busch et al., 2010; Daum et al., 2014). The mechanisms of WUS homodimerization have been recently studied in more depth (Box 1). Rodriguez et al. (2016) identified two distinct regions that are necessary for WUS dimerization. They also proposed that the dimerization could be promoted by the binding to DNA, although this may not be strictly necessary (Busch et al., 2010), and that it affects the stability of the TF (Rodriguez et al., 2016). A further study from the same team showed that the homodimerization occurs in a concentration-dependent manner and that it affects binding to target genes (Perales et al., 2016). As WUS movement through plasmodesmata is size-dependent (Yadav et al., 2011) and as one of the sequences required for homodimerization is also necessary for mobility (Rodriguez et al., 2016), the formation of dimers may reduce WUS mobility (Fuchs and Lohmann, 2020) (Box 1).WUS has been shown to bind to DNA through three different motifs: a canonical TAAT motif for homeodomain TFs, a G-box-like domain, and a TGAA domain (Lohmann et al., 2001; Yadav et al., 2011; Perales et al., 2016). Upon binding, WUS can act as an activator but also as a repressor thanks to the recruitment of co-repressors from the TOPLESS family (Leibfried et al., 2005; Busch et al., 2010). Interestingly, Perales et al. (2016) showed that the formation of homodimers could affect both the binding to DNA and the activity of WUS (Box 1). They proposed a model in which WUS monomers would activate CLV3 in the CZ but WUS dimers would repress CLV3in the OC. Sloan and colleagues recently obtained

the structure of fragments of WUS proteins bound to different DNA sequences that allowed them to study its binding specificity further (Sloan et al., 2020). They showed that WUS preferentially binds to TGAA sequences and that the dimerization allows co-operative and stabilized binding to specific repeated motifs. In addition to the formation of homodimers, WUS can also form heterodimers with members of the HAIRY MERISTEM (HAM) family of TFs (Zhou et al., 2015, 2018) (Box 1). The triple ham1.2.3 mutant has a very intriguing phenotype in which CLV3 expression increases and moves from the CZ to the OC. Given that HAM is only expressed in L3, Zhou et al.(2018) proposed that the formation of heterodimers between WUS and HAM prevents the induction of CLV3 in the OC while the absence of HAM in L1 and L2 allows the induction of CLV3 by WUS in the CZ. This model is supported by two sets of computational simulations that could recapitulate both WT and mutant phenotypes (Zhou et al., 2018; Gruel et al., 2018). Very recently, Su et al. (2020) also showed that WUS physically interacts with STM. They also demonstrated that STM could bind to the CLV3 promoter and that this binding strengthened the binding of WUS through the formation of a WUS–STM heterodimer (Box 1). Taking these studies together, we can build a model in which WUS homodimers and/or WUS–HAM heterodimers would inhibit CLV3 expression in the OC, while WUS-STM heterodimers or WUS monomers would induce CLV3 ex-pression in the CZ (Box 1). Studying the movement, DNA binding, and transcriptional activity of the WUS homodimers and heterodimers should allow us to further understand how the dimerization affects WUS function in both CZ and OC.

Box 1. Recent developments in our comprehension of *WUS* regulation of *CLV3* expression

- **WUS dimerization could explain its dual functions in the OC and in the CZ**

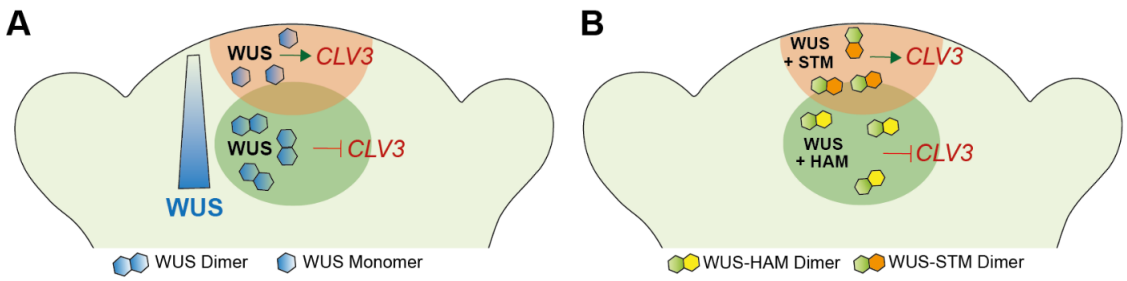
Rodriguez *et al.* (2016) and Perales *et al.* (2016) showed that WUS forms stable homodimers upon binding to DNA in a concentration-dependent, which affects its binding to the *CLV3* promoter. They proposed a model where WUS dimers negatively regulate *CLV3* expression in the OC while WUS monomers positively regulate *CLV3* expression in the CZ (panel A).

- **WUS can form heterodimers with HAM in the OC**

Zhou *et al*. (2018) showed that WUS can physically interact with members of the *HAM* family of TF, which are specifically expressed in the L3 of the SAM. They proposed a model where WUS/HAM heterodimers repress *CLV3* expression in the OC while WUS alone in the CZ induces *CLV3* expression (panel B).

- **WUS can form heterodimers with STM in the CZ**

Su *et al.* (2020) showed that WUS can physically interact with STM and that STM binding to *CLV3* promoter can enhance the stability of WUS binding to this promoter through the formation of heterodimer in the CZ (panel B).



2.4. WUS interaction with hormone signalling

Following the identification of its targets, it was shown that WUS maintains stem cell identity by repressing differentiation, and that many of the WUS targets are involved in hormone signalling (Leibfried et al., 2005; Busch et al., 2010; Yadav et al., 2013; Ma et al., 2019) (Box 2). WUS and cytokinins (CKs) have an intricate feedback loop in which WUS activates CK signalling by repressing negative regulators of CK signalling of the type-A ARABIDOPSIS RESPONSE REGULATOR (ARR) family (Leibfried et al., 2005). In return, CKs promote stem cell fate by inducing WUS expression but also by repressing CLV1 expression (Gordon et al., 2009; Buechel et al., 2010; Nimchuk et al., 2015). Interestingly, it was also proposed that the positioning of the WUS domain relies on cytokinins, and more specifically on the expression pattern of the ARABIDOPSIS HISTIDINE KINASE (AHK) cytokinin receptors (Gordon et al., 2009; Chickarmane et al., 2012) . Gruel et al. (2016) further supported this idea through computational studies. They showed that the diffusion of two mobile signals from the epidermis, one corresponding to active CKs, the other to an unknown molecule restricting expression of AHKs to the L3, could position the WUS domain in the OC. Interesting scaling properties of the system were highlighted using this model as it was shown that the WUS expression domain can scale to the size and the curvature of the SAM (Box 2). Identifying the molecule controlling expression of AHKs should allow testing of the predictions of this model and confirming that CK signalling indeed controls the positioning of the WUS domain in the OC. In addition to cytokinins, the WUS–CLV feedback loop has also been tightly connected to auxin signalling. Auxin accumulates at specific positions in the PZ to induce organ emergence thanks to polar transport mediated by PIN-FORMED 1 (PIN1) (Reinhardt et al., 2000). Although auxin also ac-cumulates

in the CZ, signalling is low in this zone, which is mostly insensitive to the hormone (Vernoux et al., 2011). A re-cent paper from Ma et al. (2019) proposed that the maintenance of the stem cells in such a low auxin signalling state is controlled by WUS (Box 2). WUS notably reduces the expression of MONOPTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5) at the CZ to lower auxin signalling. They demonstrated that WUS also directly regulates the expression of other genes involved in auxin signalling and response through histone de-acetylation, thus preventing cells at the CZ from differentiating. Interestingly, Galvan-Ampudia et al. (2020) also recently showed that newly formed auxin maxima corresponding to the future primordia are formed as protrusions originated from the CZ, but these only emerge at the boundaries of the CZ where the temporal integration of auxin concentration allows the activation of auxin signalling (Box 2). From these studies, we can build a model in which WUS maintains stem cell fate by limiting auxin signalling in the CZ through chromatin modification, restricting organ emergence to the PZ where auxin signalling can occur. Interestingly, we can hypothesize that this inhibition of auxin signalling by WUS may control the rate of organ emergence in the SAM as a result of the way organs emerge following specific patterns of phyllo-taxis in the SAM (Box 2). Auxin can also feedback on stem cell homeostasis, thus adding another loop to the system. It has indeed been shown that MP/ARF5 inhibits the expression of two negative ARRs in the CZ, which could thus induce cytokinin signalling and WUS expression in the SAM (Zhao et al., 2010). MP/ARF5 can also repress the expression of DORNROSCHEN/ENHANCER OF SHOOT REGENERATION 1 (DRN/ESR1), a positive regulator of CLV3 expression (Luo et al., 2018) (Box 2). Although transcriptional repression is not through direct binding to the CLV3 promoter, DRN/ESR1 is required to maintain CLV3 expression in the stem cells. However, the *drn/drnl* double mutant does not

recapitulate the *clv3* mutant, suggesting that DRN might help to fine-tune the extension of the stem cell niche rather than determining it

Box 2. Recent developments in our comprehension of the mechanisms of *WUS* regulation of meristem function

- **WUS modulate auxin signalling in the CZ**

Ma *et al.* (2019) showed that WUS acts as a rheostat to maintain stem cells in a low auxin signalling state by modulating the expression of many genes involved in auxin signalling and response through histone acetylation.

- **Auxin maxima are produced in the CZ but only emerge in the PZ**

Galvan-Ampudia *et al.* (2020) showed that newly formed auxin maxima corresponding to the future primordia are formed as protrusions originated from the CZ but only emerge in the PZ, which could result from the inhibition of auxin signalling by WUS in the CZ.

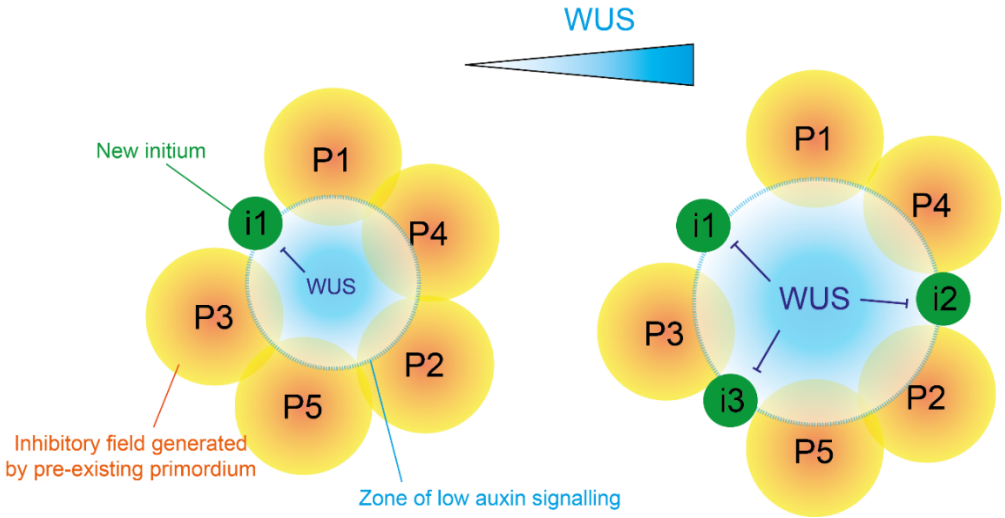
- **Auxin can feedback on stem cell regulation through MP/ARF5**

Luo et al. (2018) showed that MP/ARF5 can repress the expression of DRN/ESR1, a positive regulator of *CLV3* which, thus activating *WUS* expression and stem cell fate through the inhibition of *CLV* signalling.

- **A model for WUS control of organogenesis in the SAM**

The emergence of new organs in the SAM results from the accumulation of auxin at specific location thanks to polar auxin transport by PIN1 proteins (Reinhardt *et al.*, 2000). This mechanism of organ positioning (phyllotaxis) has long been conceptualized by the so-called inhibitory field theory (Landrein and Vernoux, 2014). This theory states that pre-existing organs inhibit the initiation of new organs at their vicinity (by depleting auxin from the surroundings).

Interestingly, theoretical work testing this theory and partly validated experimentally have shown that two key parameters could control the positioning and timing of organ emergence through inhibitory fields: the size of the inhibitory fields and the radius of the central ring on which organs are initiated (Douady and Couder, 1996; Landrein *et al.*, 2015). By combining the recent work from Ma and colleagues and from Galvan-Ampudia and colleagues, we could hypothesize that WUS may control this second parameter by inhibiting of auxin signalling. In such scenario, increasing *WUS* activity would inhibit auxin signalling locally but increase organogenesis globally (See bellow). This model, that remains to be tested, would explain the tight correlation that can be measured between *WUS* expression, meristem size (which is defined as the distance between the centre of the SAM and the organs) and organogenesis rate (Landrein *et al.*, 2015, 2018).



2.5. WUS and the transduction of environmental cues

In addition to internal signals, it has been recently shown that several environmental cues such as light levels, sugar levels, mineral nutrient availability, and oxygen levels can affect meri-stem function through hormone signalling and the CLV–WUS loop (Box 3). Light is known to modulate SAM activity through auxin and cytokinins and thus influence plant growth at several stages of development (Yoshida et al., 2011; Pfeiffer et al., 2016). Accordingly, Pfeiffer et al. (2016) demonstrated that light and metabolic signals converge towards the TARGET OF RAPAMYCIN (TOR) pathway to modulate WUS ex-pression in the SAM of germinating seedlings. They further showed that this effect is, at least partly, dependent on the activity of two CK degrading enzymes from the CYTOKININ OXIDASE (CKX) family (Box 3). Nitrogen (N) is a major mineral nutrient for plants whose availability in the soil affects metabolism, growth, and develop-mental processes (Vidal et al., 2020). The perception of N in different organs and the integration of this information through long-distance signalling are key for coping with changes in N availability. Although nitrate can act as a signal itself, one of the major long-distance N signalling pathways is mediated by CK (Zhang et al., 2020) (Box 3). SAM activity also responds to changes in nitrate availability in a cytokinin-dependent manner. Osugi et al. (2017) indeed showed that an increase in nitrate levels in the soil leads to the production of CK pre-cursors by specific ISOPENTENYL TRANSFERASE (IPT) enzymes in the root and to their translocation via the xylem to the shoot. Landrein et al. (2018) further showed that the generation of active cytokinin by LONELY GUY (LOG) enzymes from these precursors in the SAM induces CK signalling and WUS expression. This activation leads to stem cell proliferation and increased meristem size and organ production rate (Box 3). However, the effect of nitrate

on meristem function may be more complex and only partly mediated by CK signalling. Nitrate is also a major source of nitric oxide (NO), a central redox signalling molecule (Fancy et al., 2017). Interestingly, keeping the balance between the different forms of reactive oxygen species (ROS), such as NO, superoxide anion ($O2\cdot -$), or hydrogen peroxide ($H2O2$), affects growth and development in both shoots and roots and is important for SAM robustness against environmental fluctuations (Foyer et al., 2018). Hence, accumulation of ROS species in the SAM following mutation of AtFTSH4, encoding an ATP-dependent mitochondrial pro-tease that counteracts accumulation of internal oxidative stress, causes meristem termination at higher temperatures (Dolzblasz et al., 2016). Interestingly, the key enzymes regulating ROS metabolism have a distinct spatial distribution within the SAM and the different forms of ROS play distinct roles in each do-main (Yadav et al., 2009; Zeng et al., 2017; Foyer et al., 2018) (Box 3). For example, depleting $O2\cdot -$ in the CZ leads to a decrease of WUS transcript and protein levels and CLV3 ex-pression leading to meristem termination (Zeng et al., 2017). $H2O2$, on the other hand, is mainly present in the PZ, where it inhibits WUS expression and promotes stem cell differentiation (Zeng et al., 2017). $H2O2$ production can be promoted via nitrate- or cytokinin-induced NO, through the induction of superoxide dismutase, which converts superoxide ($O2\cdot -$) into $H2O2$, pointing to a possible role of NO in the regulation of stem cell activity (Wany et al., 2018). Supporting this idea, cytokinins are required for the activation of the CYCD3 cell cycle gene by NO, thereby promoting cell proliferation (Shen et al., 2013). Oxygen levels may also affect stem cell homeostasis in both plants and animals (Le Gac and Laux, 2019). Weits et al. (2019), using a microscale oxygen electrode, showed that the SAM is a closed hypoxic niche (Box 3). Consistent with this, a large number of core hypoxia-induced genes are up-regulated in the SAM when compared with juvenile leaves and their

expression is down-regulated when meristems are exposed to higher oxygen concentrations (80%) (Weits et al., 2019). Most importantly, increasing the levels of oxygen resulted in decreased leaf production rates, supporting the importance of hypoxia for SAM activity (Weits et al., 2019). Part of this response is mediated by LITTLE ZIPPER2 (ZPR2), which is degraded by the oxygen-dependent N-degron pathway and is thus stabilized at low O2 (Wenkel et al., 2007; Weits et al., 2019). Several targets of ZRP2, including HD-ZIPIII TFs and HECATE (HEC), have been shown to be involved in stem cell regulation, not-ably by modulating WUS expression. Recently, Wu et al. (2020) have shown that WUS was also involved in plant immunity, by acting as a molecular barrier against virus spreading at the shoot apical meristem. Upon viral infection, WUS protein is stabilized and actively represses the expression of methyltransferases necessary for ribosome stability, thus reducing global protein synthesis and keeping the virus out of the meristem. How WUS protein is stabilized upon viral infection is still an open question, but this work very interestingly showed a new role for WUS in protecting stem cells from infections.

Box 3. Influence of environmental signals on stem cell homeostasis in the SAM

- **Light and metabolic signals can modulate *WUS* expression in germinating seedlings**

Pfeiffer *et al.* (2016) showed that light and metabolic signals are integrated by the TOR to regulate WUS expression in the SAM of germinating seedlings. They further showed that part of this response relied on the regulation of the activity of cytokinin degrading enzymes (panel A).

- **Nitrate can modulate WUS expression through cytokinins**

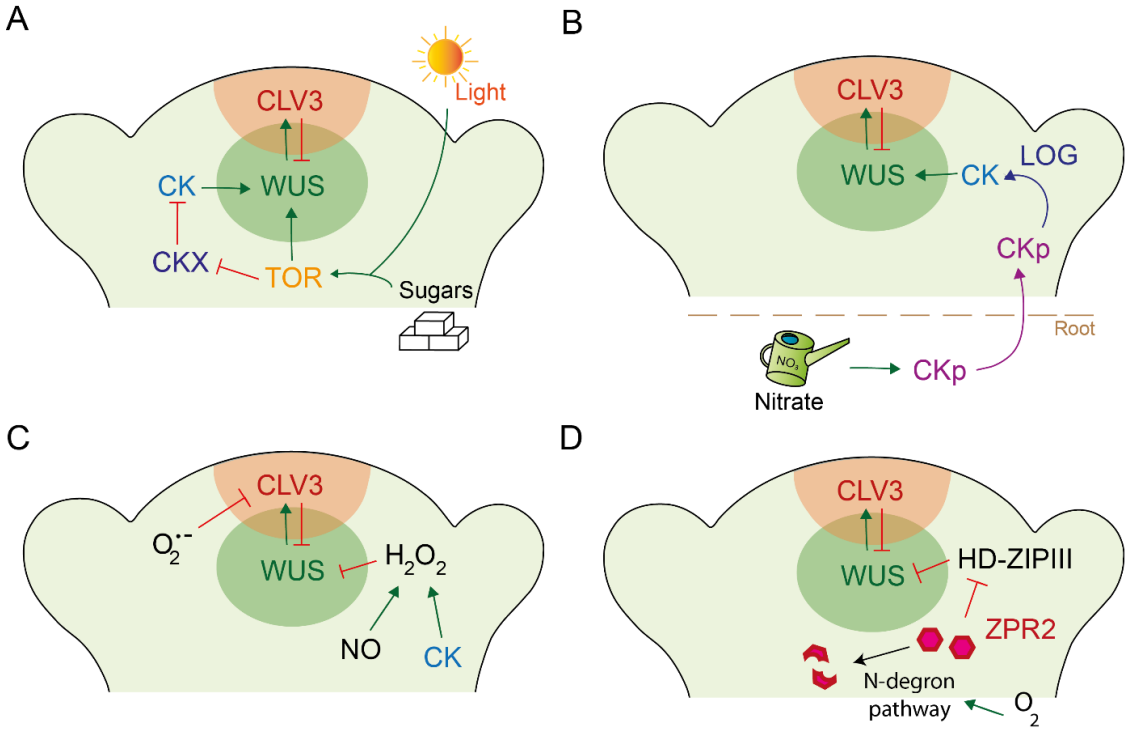
Landrein *et al.* (2018) showed that the SAM can respond to quick changes in nitrate availability in the soil thanks to long range signalling of CK precursors, that are activated in the SAM and trigger the induction of WUS expression (panel B).

- **ROS can also influence stem cell homeostasis in the SAM**

Zeng *et al.* (2017) showed that ROS-metabolizing enzymes displays specific patterns of expression in the SAM. They proposed that the balance between O2·- and H_2O_2 is involved in stem cell maintenance and differentiation in the SAM (panel C).

- **Oxygen levels can affect stem cell homeostasis in the SAM**

Weits *et al.* (2019) showed that the stem cell niche is under hypoxic conditions and that altering oxygen levels in the meristem can affect stem cell homeostasis, notably through the activity of HD-ZIPIII transcription factors. This effect is mediated through the degradation of ZPR2 by the N-degron pathway (panel D).


A
CLV3
Light
CK
WUS
CKX
TOR
Sugars
B
CLV3
WUS
CK
LOG
CKp
Root
Nitrate
CKp
C
CLV3
$O_2^{\bullet-}$
WUS
H_2O_2
NO
CK
D
CLV3
WUS
HD-ZIPIII
ZPR2
N-degron
pathway
O_2

2.6. Conclusions

Accumulating evidence gathered in recent years has high-lighted WUSCHEL as a central regulator of stem cell fate and differentiation in the SAM and as a point of convergence for both internal and external signals. The recent characterization of the activity of WUS homodimers and heterodimers with HAM and STM has been a huge step forward in our understanding of WUS function. Thanks to that, we can hypothesize that WUS activity in distinct tissues (such as the OC and CZ), organs, and stages of development might rely on the formation of specific heterodimers with other TFs, including HAM and STM. The study of the interplay between the WUS–CLV loop and auxin and cytokinins also highlights very strong connections between stem cell maintenance and hormone signalling. This work notably allowed redefinition of the function of WUS in controlling organogenesis through the inhibition of differentiation. Finally, the recent characterization of the impact of environmental signals on WUS expression showed that the CLV–WUS feedback loop is finely tuned to adapt meristem function to a variety of signals, and that hormones play a central role in this process. A key question that remains to be answered is how all of these signals can be integrated by both hormone signalling and WUS for plants to adapt meristem maintenance and organogenesis to their constantly changing local environment.

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PTDC/BIA-BID/32347/2017). The work of CG-A is supported by the Agence Nationale de la Recherche (ANR-18-CE13-0019 2018 to Teva Vernoux).

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CHAPTER III

Sugar signalling modulates SHOOT MERISTEMLESS expression and meristem function in Arabidopsis

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Author contributions: Filipa Lopes, Benoît Landrein, Henrik Jönsson and Elena Baena-González conceived the research plan and designed the experiments. Filipa Lopes, Alice Malivert and Benoît Landrein performed the experiments. Regina Feil and John Lunn performed the metabolite profiling. Pau Formosa-Jordan developed the custom-made code written in Matlab (Mathworks Inc., Natick, MA) here described for the data analysis. Filipa Lopes, Benoît Landrein and Elena Baena-González analysed and interpreted data.

3.1. Abstract

In plants, development of all above-ground tissues is controlled by the shoot apical meristem (SAM) which finely balances cell proliferation and differentiation to allow life-long growth. To maximize fitness and survival, meristem activity is adjusted to the prevailing conditions through a poorly understood integration of developmental signals with environmental and nutritional information. Here, we show that sugar signals play a central role in SAM function through changes in the levels of the key regulator of meristem maintenance, SHOOT MERISTEMLESS (STM). STM protein is less abundant in the inflorescence meristem of plants grown under limiting light conditions or transferred from optimal irradiance to darkness for up to three days, with lower STM expression correlating with lower sugar content in these meristems compared to control plants. We further show that STM accumulation requires sucrose rather than light *per se*. Subjecting plants to prolonged darkness activates the sugar sensing protein kinase SUCROSE-NON-FERMENTING1-RELATED KINASE 1 (SnRK1) in the SAM, where SnRK1 is particularly abundant. Plants overexpressing SnRK1α1 have decreased STM levels under optimal light conditions, despite a higher sugar accumulation in the SAM. In contrast, *SnRK1α* silencing in the meristem leads to reduced *STM* expression and severe developmental phenotypes previously associated with STM loss-of-function. Altogether, we demonstrate that sugars promote STM accumulation and that the SnRK1 sugar sensor plays a dual role in the SAM, limiting STM abundance under unfavourable conditions but being required for overall meristem organization and integrity. This highlights the importance of sugar signalling for the proper coordination of meristem activities.

3.2. Introduction

Unlike animals, plants generate organs throughout their life cycle. Postembryonic development relies on stem cell reservoirs localized in specialized tissues known as meristems. The shoot apical meristem (SAM) is the site where above-ground organogenesis is initiated, giving rise to leaves, axillary buds, flowers, and the stem. The SAM is organized into functionally distinct subdomains in which cell division and differentiation are tightly coordinated to maintain the integrity and regenerative potential of the meristem. The concerted regulation of the different regions requires the activity of several transcriptional networks and hormone signalling pathways (Barton, 2010).

In Arabidopsis (*Arabidopsis thaliana*), SAM homeostasis is finely controlled by a negative-feedback loop between WUSCHEL (WUS) and CLAVATA3 (CLV3) (Brand et al., 2000; Schoof et al., 2000). WUS is a mobile transcription factor expressed in the organizing center (OC) that underlies the stem cell layers. WUS moves through plasmodesmata into the overlying stem cells to promote their proliferation and maintain pluripotency (Daum et al., 2014; Yadav et al., 2011). In addition, WUS induces stem cells to produce the CLV3 peptide, which in turn diffuses into the OC, where it inhibits *WUS* expression. The WUS-CLV3 feedback loop therefore enables a dynamic adjustment of the size of the stem cell pool (Williams & Fletcher, 2005).

Meristematic activity is also regulated by members of the THREE-AMINO-ACID-LOOP-EXTENSION (TALE) homeodomain protein superfamily which includes KNOTTED1-like homeobox (KNOX) and BEL-like homeobox (BLH or BELL) transcription factors. One key regulator of the TALE family is SHOOT MERISTEMLESS (STM), which is essential both for SAM establishment and SAM maintenance (J. A. Long et al., 1996). Unlike WUS, STM is expressed

throughout the SAM except at the sites of initiating primordia (J. A. Long et al., 1996; J. Long & Barton, 2000). STM suppresses differentiation independently of WUS and promotes cell division by inducing the expression of *CYCLIN D3* (*CYCD3*) and *ISOPENTENYL TRANSFERASE7* (*IPT7*) (Scofield et al., 2013), which encodes a key enzyme involved in cytokinin (CK) biosynthesis (Yanai et al., 2005). CKs, in turn, are involved in stem cell maintenance, influencing SAM size and organ production through WUS and STM (Gordon et al., 2009; Landrein et al., 2018; Rupp et al., 1999).

Loss-of-function *stm* mutants exhibit defects in SAM formation and maintenance, leading to growth arrest at the seedling stage due to exhaustion of stem cells (J. A. Long et al., 1996). In addition, the most severely affected mutants like *stm-1* display fusions of cotyledons and other organs, indicating a role for STM also in boundary specification (Clark et al., 1996; Endrizzi et al., 1996; J. A. Long et al., 1996). Indeed, incipient organ primordia are formed at sites where *STM* expression is low, coincident with auxin accumulation and the expression of transcription factors (TFs) that promote organ formation and repress *STM*, including ASYMMETRIC LEAVES1 and 2 (AS1 and AS2) and members of the TEOSINTE BRANCHED1/CYCLOIDEA/PCF1 (TCP) family (Aguilar-Martlnez & Sinha, 2013; Benková et al., 2003; Heisler et al., 2005; Z. Li et al., 2012; Reinhardt, 2000). In the inflorescence meristem, *STM* expression is also enhanced in boundaries, notably in response to mechanical forces, and is required for correct boundary folding (Landrein et al., 2015).

Recent work revealed that STM heterodimerizes with WUS, enhancing WUS binding to the *CLV3* promoter and *CLV3* expression, and repressing stem cell differentiation. Conversely, WUS is required for the expression of *STM,* which thereby enhances WUS-mediated stem cell activity (Su et al., 2020). STM is also regulated through an interaction with BELL1-like homeodomain (BLH) proteins (Hake et al., 2004) and the formation of these

heterodimeric complexes is essential for their nuclear localization and, thus, proper function (Cole et al., 2006). STM interacts with the BLH proteins PENNYWISE (PNY), POUNDFOOLISH (PNF), and ARABIDOPSIS THALIANA HOMEOBOX GENE1 (ATH1) (Bhatt et al., 2004; Byrne et al., 2003; Cole et al., 2006; Smith & Hake, 2003) which contribute redundantly with STM to meristem initiation and maintenance (Belles-Boix et al., 2006; Byrne et al., 2000; Rutjens et al., 2009).

Because of their sessile lifestyle, plants continuously adjust their development to changes in the environment, and this is reflected in the dynamic nature of the SAM. In addition to its maintenance by a network of TFs and hormonal signals, the SAM also responds to environmental signals that influence the relative size of its subdomains and the type and number of organs it produces. One of the external factors that affect meristem activity is light, which can exert a direct effect through photoreceptor-mediated signalling and an indirect effect by driving photosynthesis and sugar production (Pfeiffer et al., 2016, 2017; Yoshida et al., 2011). Both light and metabolic signals activate the TARGET OF RAPAMYCIN (TOR) protein kinase, which in turn promotes cell proliferation in the SAM *via* an increase in the expression of S-phase genes (X. Li et al., 2017; Xiong et al., 2013). In addition, TOR induces *WUS* expression partially through an effect on CK degradation (Janocha et al., 2021; Pfeiffer et al., 2016).

TOR activity is often antagonized by SUCROSE NON-FERMENTING1-RELATED KINASE 1 (SnRK1) which, like TOR, translates environmental information into metabolic and developmental adaptations (Baena-González & Hanson, 2017; Jamsheer et al., 2021; Margalha et al., 2019). SnRK1 is a heterotrimeric protein kinase complex, composed of an α-catalytic subunit and two regulatory β- and γ-subunits. In Arabidopsis, the α-subunit is present in two major isoforms, SnRK1α1 and SnRK1α2 (also known as KIN10 and

KIN11). The SnRK1 complex is activated under low carbon conditions to promote energy saving and nutrient remobilization strategies, whilst TOR is activated in response to nutrient abundance to promote cell proliferation and growth adaptations (Baena-González & Hanson, 2017; Jamsheer et al., 2021; Margalha et al., 2019).

Despite the importance of STM for establishing and maintaining SAM function and our increasing understanding of how STM activity is controlled by other transcriptional regulators, it is unknown if environmental signals modulate STM expression and/or activity. Here, we make use of plants expressing transcriptional and translational STM reporters to investigate this question. We show that STM protein accumulation does not respond to CK but is clearly induced by photosynthesis-derived sugars. We also show that the SnRK1 sugar sensing kinase is active in the SAM and that it is involved in the regulation of STM protein accumulation in response to light. Finally, we show that SnRK1 is also required to maintain *STM* expression, meristem organization and integrity.

3.3. Results

3.3.1. Light promotes STM protein accumulation

Light is essential for proper plant development and physiology. To investigate a potential regulatory role of light on STM levels, we made use of an Arabidopsis (Col-0) reporter line in which a fluorescently-tagged form of the STM protein (STM-VENUS) is expressed under the control of the *STM* promoter [*pSTM::STM-VENUS* (Heisler et al., 2005; Landrein et al., 2015)]. We measured STM-VENUS levels in inflorescence meristems from 5-week-old plants constantly grown under, or transiently treated with different light conditions. In one set of experiments, we compared STM-VENUS levels between plants grown under two different irradiances [60 *vs*. 170 µmol m^{-2} s^{-1}, referred to as low light (LL) and high light (HL), respectively]. Irradiance had a strong impact on STM accumulation, with the mean STM-VENUS levels of plants grown under LL being 76% of those grown under HL (Fig. 1A-B). In a second set of experiments, we compared STM-VENUS levels between HL-grown plants transferred to darkness for up to 72 h and their corresponding controls maintained under HL conditions. Incubation under darkness had a very severe impact on STM accumulation, with STM-VENUS levels of plants subjected to a 72h dark treatment being 39% of those prior to the treatment (Fig. 1A, 1C).

To assess whether the impact of light on STM levels was a general effect on protein abundance in meristems, we extracted total proteins from SAMs of plants constantly grown under or transiently treated with different light conditions and compared STM levels to those of the housekeeping protein TUBULIN (TUB) by immunoblotting (Fig. 1D). These analyses confirmed the microscopy results regarding STM-VENUS accumulation (71% and 23% of the levels in HL for LL-grown and dark-treated plants, respectively)

but revealed no impact of the light conditions on TUB accumulation, indicating that the lower STM levels were not caused by a general decrease in protein accumulation. Finally, to assess if low STM accumulation could be due to reduced *STM* transcript abundance, we dissected SAMs of plants kept under control HL conditions or subjected to the most severe treatment (48 h darkness) and analysed *STM* transcript levels by quantitative RT-PCR (qPCR). *STM* levels were not significantly affected by the dark treatment (Fig. 1E), showing that the differences in protein accumulation are caused by regulatory mechanisms that act at the protein level. On the other hand, the levels of *AINTEGUMENTA-LIKE 7* (*AIL7*) and *HOMEOBOX PROTEIN 25* (*HB25*), which are known gene targets of STM (Scofield et al., 2018), were reduced upon the dark treatment (Fig. 1E). This is consistent with the lower STM-VENUS abundance observed in these conditions and indicates decreased STM activity in the SAM.

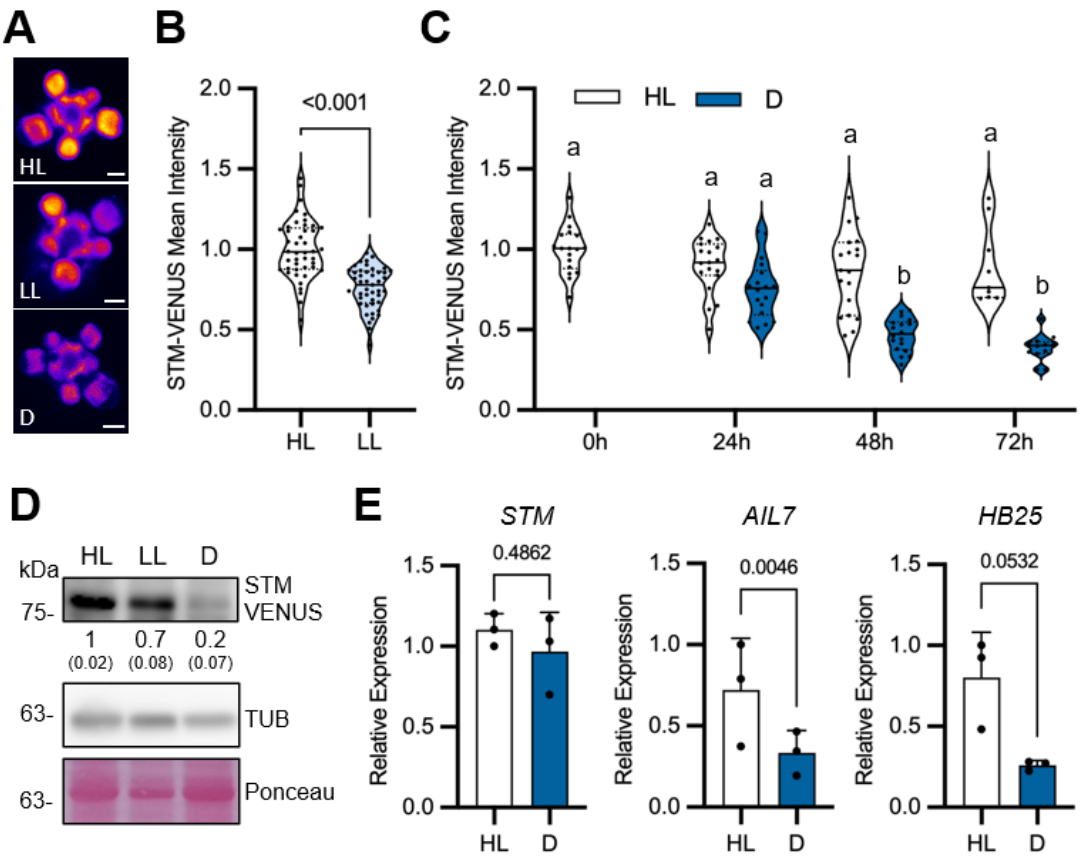


Figure 1. Effect of light on STM expression. A-C, STM-VENUS expression in SAMs of *pSTM::STM-VENUS* plants grown under high light (HL) or low light (LL) conditions or transferred from HL to darkness (D) or kept under HL for the indicated times. **A**, Representative STM-VENUS images of SAMs from HL and LL-grown plants and of plants transferred to D for 48 h. Scale bar, 50 µm. **B, C**. Quantification of STM-VENUS signal. **B,** Plots show SAM measurements of plants grown as 3 independent batches normalized by the mean of the HL condition of each batch (HL, *n*=44; LL, *n*=45). Student's *t*-test (*p*-value shown). **C,** Plots show SAM measurements of plants grown as 2-3 independent batches normalized by the mean of the HL condition of each batch (0h, *n*=18; 24 h L, *n*=19; 24 h D, *n*=18; 48 h L, *n*=19; 48 h D, *n*=18; 72 h L, *n*=9; 72 h D, *n*=12). Different letters indicate statistically significant

differences (Kruskal-Wallis with Dunn´s test; $p<0.05$). **D,** Immunoblot analyses of STM protein levels in SAMs of plants grown under HL or LL conditions or grown in HL and transferred to D for 48 h. TUBULIN (TUB) and Ponceau staining serve as loading controls. Numbers refer to mean STM-VENUS amounts in LL and D as compared to HL ($n=2$; each a pool of 5 SAMs; in parenthesis, SEM). **E,** RT-qPCR analyses of *STM* and STM target genes *AIL7* and *HB25* in SAMs of *pSTM::STM-VENUS* plants grown in HL and transferred to D or kept in HL for 48 h. Graphs show the average of 3 independent samples, each consisting of a pool of 5 SAMs. Paired ratio *t*-test (*p*-values shown).

3.3.2. The response of STM to light is CK-independent and involves sugars

Several lines of evidence suggest that, like *WUS*, *STM* expression could be directly regulated by CK (Rupp et al., 1999; Teo et al., 2001). To investigate whether CK could also regulate STM at the protein level and hence be involved in the response of STM to light, we first tested whether light influenced CK signalling in inflorescence meristems. To this end we used plants expressing the synthetic CK reporter *pTCSn::GFP* (Zürcher et al., 2013) in similar experiments as for the *STM-VENUS* reporter line. In plants grown in LL or subjected to a 48 h dark treatment, *pTCSn::GFP* levels were 74% (Supplementary Fig. S1A-B) and 46% (Supplementary Fig. S1A, S1C) of those in HL control plants, respectively. These observations show that CK signalling in inflorescence meristems is, like in vegetative meristems (Pfeiffer *et al*, 2017), affected by light. Having established that CK signalling is differentially affected by our light growth conditions and treatments, we next

examined whether CK could impact STM levels in inflorescence meristems. For this, we excised SAMs of HL-grown *STM-VENUS* plants and grew them *in vitro* (Landrein et al., 2018) for different periods of time in the absence or presence of 500 nM 6-benzylaminopurine (BAP), a synthetic CK. Dissection of the SAMs led to a strong reduction of the STM-VENUS (Supplementary Fig. S1D) and the *pTCSn::GFP* (Supplementary Fig. S1E) reporter signals, as previously described for the *pTCSn::GFP* and *pWUS::GFP* reporters (Landrein et al., 2018). However, in contrast to *pTCSn::GFP* [Supplementary Fig. S1E; (Landrein et al., 2018)], CK could not sustain STM-VENUS levels (Supplementary Fig. S1D), indicating that the effect of light on STM-VENUS, which involves changes at the protein level (Fig. 1), is CK-independent.

Light plays direct signalling functions through various photoreceptors but also signals indirectly through sugars produced by photosynthesis. We therefore wondered whether the effect of light on STM was direct or mediated by sugars. To investigate this, we first measured the levels of sucrose, glucose, and fructose in the rosettes (Supplementary Fig. S2A) and SAMs (Fig. 2A) of HL- and dark-treated plants. We also measured the levels of Tre6P, a regulatory sugar-phosphate that serves as a signal of the plant's sucrose status and that is crucial for sucrose homeostasis, growth promotion, and developmental progression (Fichtner & Lunn, 2021). In the light, the levels of all sugars except fructose were higher in the SAM (Fig. 2A) than in the rosette (Supplementary Fig. S2A). Incubation in the dark led to a marked depletion of sucrose and fructose both in rosettes (15% and 11% of the levels in the light, respectively) and SAMs (8% and 4% of the levels in the light, respectively), with a much milder reduction being observed for glucose, the most abundant sugar in the SAM (44% and 35% of the levels in the light in rosettes and SAMs, respectively). Tre6P levels were also much lower in dark-treated plants (11% and 3% of the levels in the light in rosettes and SAMs,

respectively), reflecting the drop in sucrose levels. To further distinguish between a light and a sugar effect, we excised inflorescence stems at around 3 cm from the apex and placed them for 48 h in liquid medium. Similarly to what was observed in dissected SAMs (Supplementary Fig. S1D), excision of the stems caused a marked decrease in STM-VENUS signal compared to that of uncut stems (Fig. 2B). Furthermore, light alone was not sufficient to sustain STM-VENUS expression, as the signal was comparable in excised stems incubated in the light and in the dark (47% and 43% of the levels in the uncut control, respectively). Similar results were obtained for plants harbouring a *pSTM::TFP-N7* transcriptional reporter in addition to the STM-VENUS protein (Fig. 2B) and for plants expressing STM-VENUS alone (Supplementary Fig. S2B).

To test if the underlying cause was sugar deprivation, we first incubated excised stems for 48 h in darkness in medium supplemented with increasing concentrations of sucrose. Sorbitol, which is not a readily metabolized carbon source, was used as an osmotic control. Sucrose was able to sustain STM-VENUS expression, and its effect was dose-dependent, leading to STM-VENUS levels close to those of uncut SAMs when supplied at a 5% concentration (72% of the uncut control values as compared to 18% in the corresponding 2.5% sorbitol control; Fig. 2C). STM-VENUS levels did not increase in response to sorbitol, indicating that the effects of sucrose were not osmotic. Similar results were obtained for the STM-VENUS reporter line (Fig. S2C). To test if the observed effects were transcriptional, we monitored the activity of the *pSTM::TFP-N7* transcriptional reporter. Quantification of the *pSTM::TFP-N7* signal revealed only minor effects on *STM* promoter activity upon inflorescence excision and dark incubation (Supplementary Fig. S2D), consistent with the results obtained by qRT-PCR (Fig. 1E). In addition, incubation in sucrose or sorbitol-containing media had minor effects on TFP

levels (Supplementary Fig. S2E) as compared to STM-VENUS (Fig. 2C), with the most severe condition (2.5% sorbitol) leading to 65% of the signal in the uncut control as compared to the 18% of the equivalent STM-VENUS samples. Altogether, this indicates that the effect of sugar deprivation, like the effect of limiting light conditions, is at the protein rather than at the transcript level.

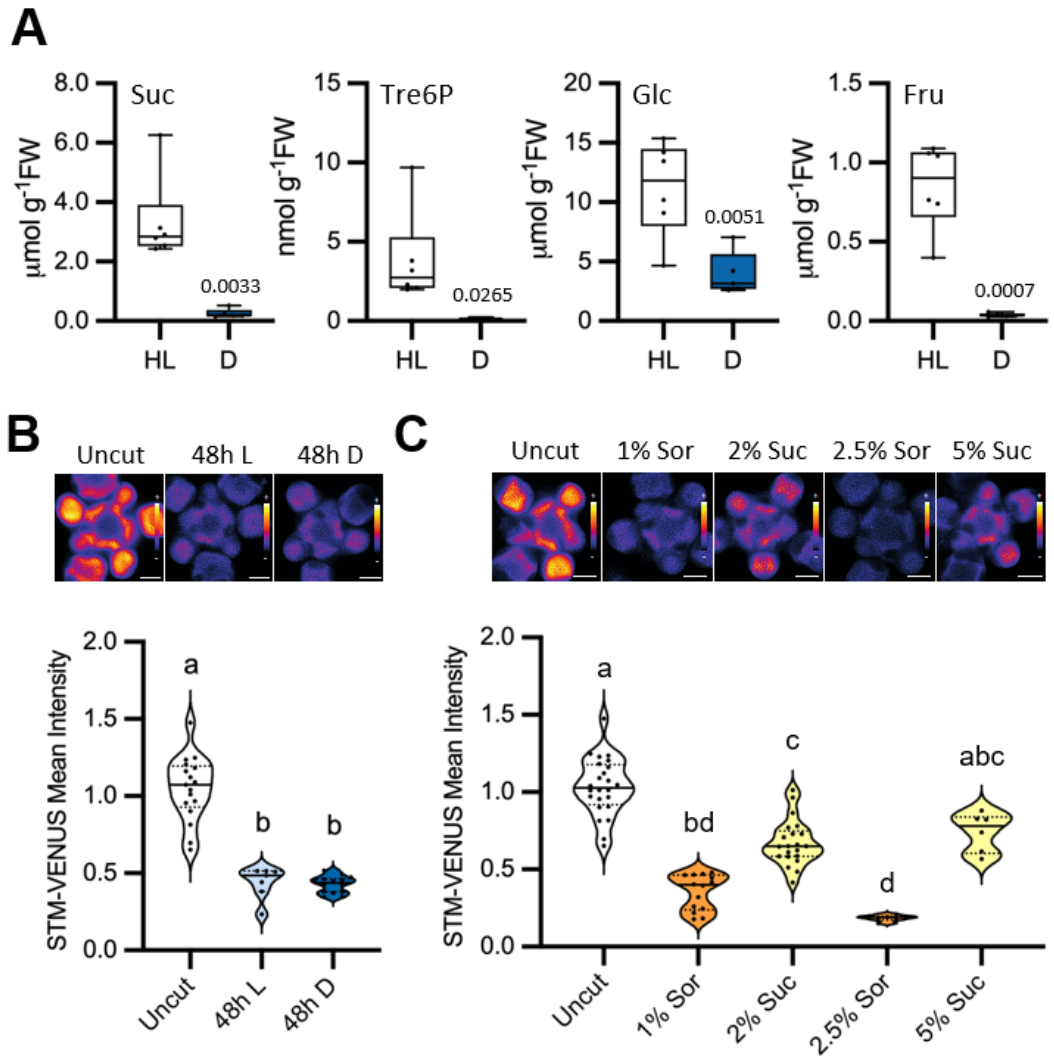


Figure 2. Effect of sugars on STM levels. A, Effect of light on the levels of soluble sugars in SAMs of *pSTM::STM-VENUS* plants grown in high light (HL) and transferred to darkness (D) or kept in HL for 48 h. Suc, sucrose; Tre6P, trehalose 6-phosphate; Glc, glucose; Fru, fructose. Plots show measurements of 5-6 samples, each consisting of a pool of 5 SAMs from plants grown as 2 independent batches. Welch´s *t*-test (*p*-value shown). **B,** Effect of light on STM-VENUS levels in cut inflorescences. Inflorescences of *pSTM::STM-VENUS/pSTM::TFP-N7* plants grown under HL were cut and placed in medium without sugar for 48 h under HL (L) or dark (D) conditions, after which the SAMs were dissected and imaged (VENUS). Upper panel, representative STM-VENUS images of SAMs. Scale bar, 50 µm. Lower panel, plots showing SAM measurements of plants grown as 1-2 independent batches normalized by the mean of the uncut condition of each batch (uncut, n=31, 2 batches; 48 h L, n=14, 1 batch; 48 h D, n=21, 2 batches). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; p<0.05). **C,** Effect of sugar on STM-VENUS levels in cut inflorescences. Inflorescences of *pSTM::STM-VENUS/pSTM::TFP-N7* plants grown under HL condition were cut and placed under darkness for 48 h in medium with sucrose (Suc; 2% and 5%) or sorbitol (Sor; 1% and 2.5%) as osmotic control. SAMs were thereafter dissected and imaged (VENUS). Upper panel, representative STM-VENUS images of SAMs. Scale bar, 50 µm. Lower panel, plots showing SAM measurements of plants grown as 1-3 independent batches normalized by the mean of the uncut condition of each batch (uncut, n=31, 3 batches; 1% Sor, n=21, 2 batches; 2% Suc, n=28, 3 batches; 2.5% Sor, n=7, 1 batch; 5% Suc, n=6, 1 batch). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; p<0.05). The same batches of HL-grown uncut plants served as controls for the experiments shown in (**B)** and (**C)**.

3.3.3. The SnRK1 sugar sensor is expressed in the SAM and influences STM levels

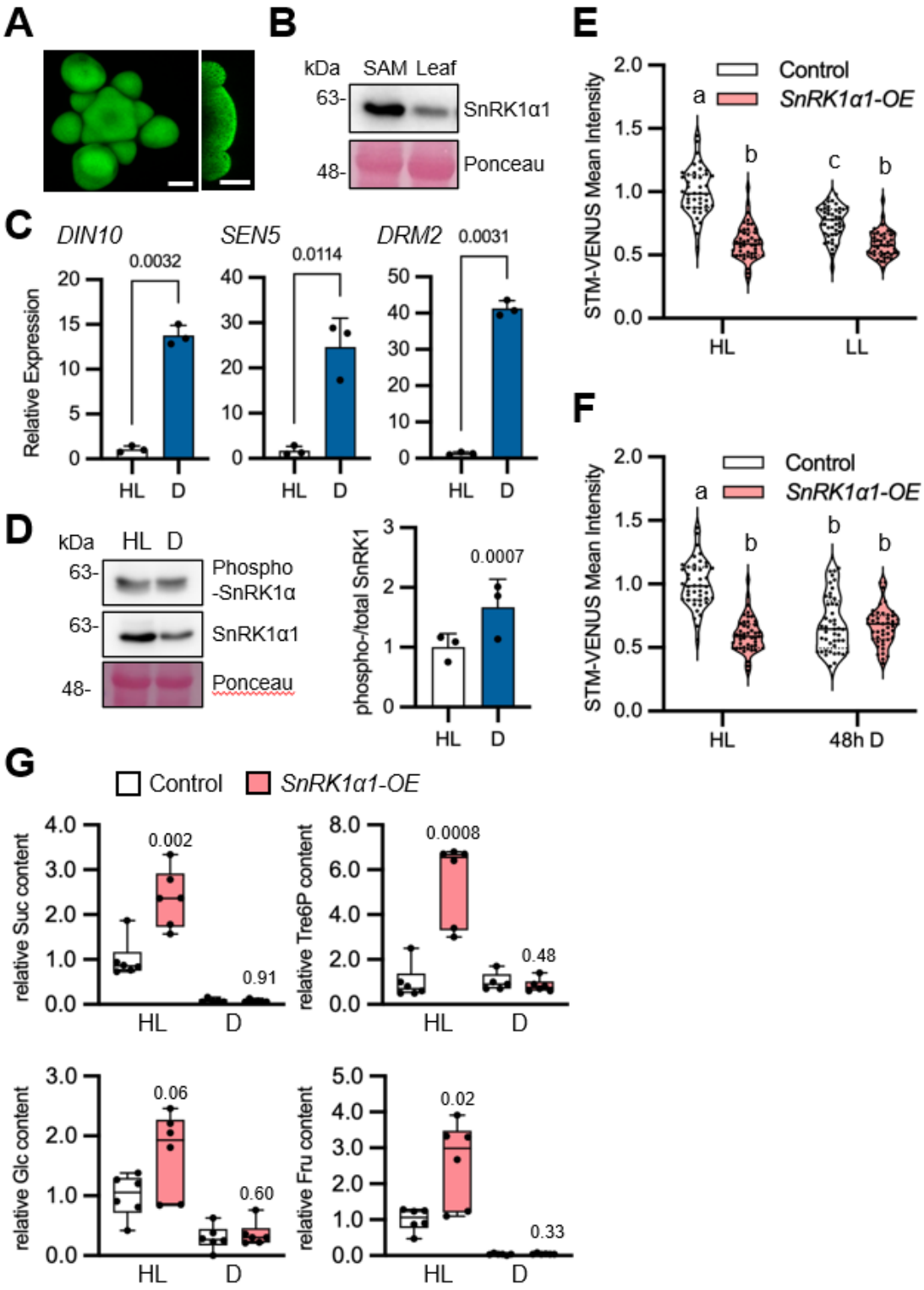
One major component of sugar signalling is the SnRK1 protein kinase, that is activated under conditions of low energy and is conversely repressed by sugars (Baena-González et al., 2007). Given its well-established role as a sugar sensor and the increasing number of studies implicating SnRK1 in developmental processes (Baena-González & Hanson, 2017; Jamsheer et al., 2021), we next investigated whether SnRK1 could be involved in the regulation of SAM function through STM. To this end, we used a line expressing SnRK1α1-GFP under the control of the *SnRK1α1* promoter and other gene regulatory regions (Crozet et al., 2016). A clear SnRK1α1-GFP signal was detected in the SAM, showing a stronger intensity in the peripheral regions, and developing organs (Fig. 3A). To further confirm this expression and to assess whether SnRK1α1 might be enriched in the SAM and surrounding floral meristems, relative to other organs, we extracted total proteins from rosettes and shoot apices of 6 to 7-week-old plants and compared the relative levels of SnRK1α1 by immunoblotting (Fig. 3B). For the same amount of total protein, shoot apices contained higher amounts of SnRK1α1 suggesting that indeed SnRK1 is more abundant in the SAM than in differentiated tissues.

To test if SnRK1 is functional in the meristem, we used SAMs dissected from HL- or dark-treated plants (48 h) to measure the activity of the SnRK1 signalling pathway using the expression of downstream target genes (Baena-González et al., 2007) as a readout (Fig. 3C). Under control conditions, SnRK1-regulated genes were barely expressed, consistent with the pathway being inactive. However, after 48 h of darkness, a marked upregulation of

these genes was observed (Fig. 3C), indicating an activation of SnRK1 signalling in the SAM. The induction of SnRK1-regulated genes was accompanied by an increase in the relative phosphorylation of the SnRK1α1 T-loop (Fig. 3D) that is essential for SnRK1 activity (Baena-González et al., 2007).

To investigate whether SnRK1 is involved in STM regulation, we introgressed the *pSTM::STM-VENUS* reporter construct into a line overexpressing SnRK1α1 [*35S::SnRK1α1*, hereafter referred to as *SnRK1α1-OE*; (Jossier et al., 2009)] and monitored STM-VENUS levels in different light conditions. When plants were grown under HL, the levels of STM-VENUS in the *SnRK1α1-OE* were 60% of those in control plants (Fig. 3E), and this could not be explained by differences in *STM* transcript levels (Supplementary Fig. S3). However, the differences between the two genotypes became smaller when plants were grown in LL (STM-VENUS levels in *SnRK1α1-OE* were 77% of those in control plants; Fig. 3E) and negligible when subjected to a 48 h dark treatment (STM-VENUS levels in *SnRK1α1-OE* were 97% of those in control plants; Fig. 3F). STM-VENUS levels thus appeared to be constitutively low and largely insensitive to the light conditions in *SnRK1α1-OE* plants. This contrasted with control plants which, in response to restrictive light conditions, reduced STM-VENUS accumulation to levels equivalent to those of the *SnRK1α1-OE.* Lower STM-VENUS levels in the *SnRK1α1-OE* in HL could not be explained by lower sugar accumulation, as these plants had a higher content of sucrose, glucose, and fructose both in the SAM (Fig. 3G) and rosettes (Supplementary Fig. S4), although the differences were not always statistically significant due to the large variation of the *SnRK1α1-OE* samples. Also, the levels of Tre6P, known to inhibit SnRK1 activity (Nunes et al., 2013; Zhai et al., 2018; Zhang et al., 2009), were markedly higher in the *SnRK1α1-OE* SAMs (5.6-fold) and rosettes (5-fold), consistent with previous

observations in *SnRK1α1-OE* rosettes (Peixoto et al., 2021). During the dark treatment, however, all sugars were largely depleted, reaching similarly low levels in control and mutant samples. Altogether these results suggest that SnRK1 is active in the SAM and that it is involved in the regulation of STM protein accumulation, potentially inhibiting it when sugar levels decline.


A
B
kDa SAM Leaf
63-
SnRK1α1
48-
Ponceau
C
DIN10
SEN5
DRM2
0.0032
0.0114
0.0031
Relative Expression
HL
D
D
kDa HL D
63-
Phospho -SnRK1α
63-
SnRK1α1
48-
Ponceau
phospho-/total SnRK1
0.0007
E
Control
SnRK1α1-OE
STM-VENUS Mean Intensity
a
b
c
b
HL
LL
F
Control
SnRK1α1-OE
STM-VENUS Mean Intensity
a
b
b
b
HL
48h D
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Control
SnRK1α1-OE
relative Suc content
0.002
0.91
relative Tre6P content
0.0008
0.48
relative Glc content
0.06
0.60
relative Fru content
0.02
0.33
HL
D

Figure 3. SnRK1 is expressed in the SAM and affects STM response to light. A, SnRK1α1-GFP imaging in the SAM. Right panel, SAM longitudinal section. Scale bars, 50 µm. **B,** Immunoblot analyses of SnRK1α1 in SAMs and rosette leaves of *pSTM::STM-VENUS* plants grown under high light (HL). 35 µg of total protein was loaded from SAM and leaf extracts. Ponceau staining serves as loading control. Similar results were obtained from two independent experiments. **C,** RT-qPCR analyses of SnRK1 signalling marker genes (*DIN10, SEN5, DRM2*) in SAMs of *pSTM::STM-VENUS* plants grown in HL and transferred to darkness (D) or kept in HL for 48 h. Graphs show the average of 3 independent samples, each consisting of a pool of 5 SAMs. Paired ratio *t*-test (*p*-values shown). **D,** Left, representative immunoblot of SnRK1α1 T-loop phosphorylation in SAMs of the plants described in **(C),** using antibodies recognizing the T175 phosphorylation (phospho-SnRK1α) or the total SnRK1α1 protein. Right, quantification of the mean SnRK1α phosphorylation (phospho-SnRK1α/total SnRK1α1) in D as compared to the ratio in HL (n=3; each a pool of 5 SAMs). Paired ratio *t*-test (*p*-value shown). **E-F,** STM-VENUS expression in SAMs of control and *SnRK1α1-OE* plants grown under HL or low light (LL) conditions (**E**) or grown under HL and transferred to darkness (D) or kept under HL for 48 h (**F**). The same batches of HL-grown plants served as controls for the experiments shown in (**E)** and (**F)**. Control HL and LL-grown STM-VENUS samples are replotted from Fig. 1B as a reference. Plots show SAM measurements of plants grown as 3 independent batches normalized by the mean of the control HL condition of each batch (control, HL: n=44, LL: n=45, D: n=45; *SnRK1α1-OE*, HL: n=45, LL: n=45; D: n=45). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; $p<0.05$). **G,** Effect of light on the levels of sugars in SAMs of *SnRK1α1-OE* plants as compared to the control. Suc, sucrose; Tre6P, trehalose 6-phosphate; Glc, glucose; Fru, fructose. Plots

show measurements of 5-6 samples, each consisting of a pool of 5 SAMs from plants grown as 2 independent batches. Welch's *t*-test (mutant *vs*. control for each condition; *p*-values shown).

3.3.4. Silencing *SnRK1α* in the SAM reduces STM expression and disrupts meristem function

The expression and relative abundance of SnRK1α in the SAM (Fig. 3A-B), together with the induction of SnRK1 signalling in apices of dark-treated plants (Fig. 3C), suggested that SnRK1 may modulate STM levels and thereby meristem function locally in the SAM. To investigate this further, we designed artificial microRNAs (amiRNAs) targeting both *SnRK1α1* and *SnRK1α2* in two different regions of the transcripts (*amiRα-1* and *amiRα-2*) and expressed these *amiRNAs* under the 5.7 kb promoter of *STM* in *STM-VENUS* plants (Fig. 4A). Immunoblot analyses confirmed a decrease in the activated form (phosphorylated in the T-loop) of SnRK1α in all lines, but this was accompanied by a decrease in total SnRK1α1 levels only in lines expressing *amiRα-2* (Fig. 4B). It is important to note that, at least SnRK1α1 is expressed throughout the apex and floral organs (Fig. 3A) and hence its silencing in the STM domain is likely to be masked by its expression in the surrounding tissues and organs.

To our surprise, depletion of SnRK1α resulted in decreased STM-VENUS accumulation, both at the protein (Fig. 4C-E) and transcript levels (Supplementary Fig. S5). The decrease in STM-VENUS levels was strongest in the lines with more compromised SnRK1α activity (*amiRα-2* lines) and weaker in the lines where the impact on SnRK1α activity was moderate or negligible (*amiRα-1* lines; Fig. 4B-E). Lower STM accumulation in the SAM of

the *SnRK1α* amiRNA lines correlated with defects in SAM development, including altered phyllotaxy, reduced bulging and the appearance of bract-like structures in some floral meristems, as well as fusions between adjacent floral meristems (Fig. 4F). Organ fusions were also visible later in development and affected cauline and rosette leaves, petals, siliques, and stems (Fig. 5D,F). Consistent with a previous report (Landrein et al., 2015), STM depletion caused defects in boundary formation, manifested as a decreased curvature at the boundary between the meristem and the incipient organ in the strong *amiRα-2* lines (Supplementary Fig. S6).

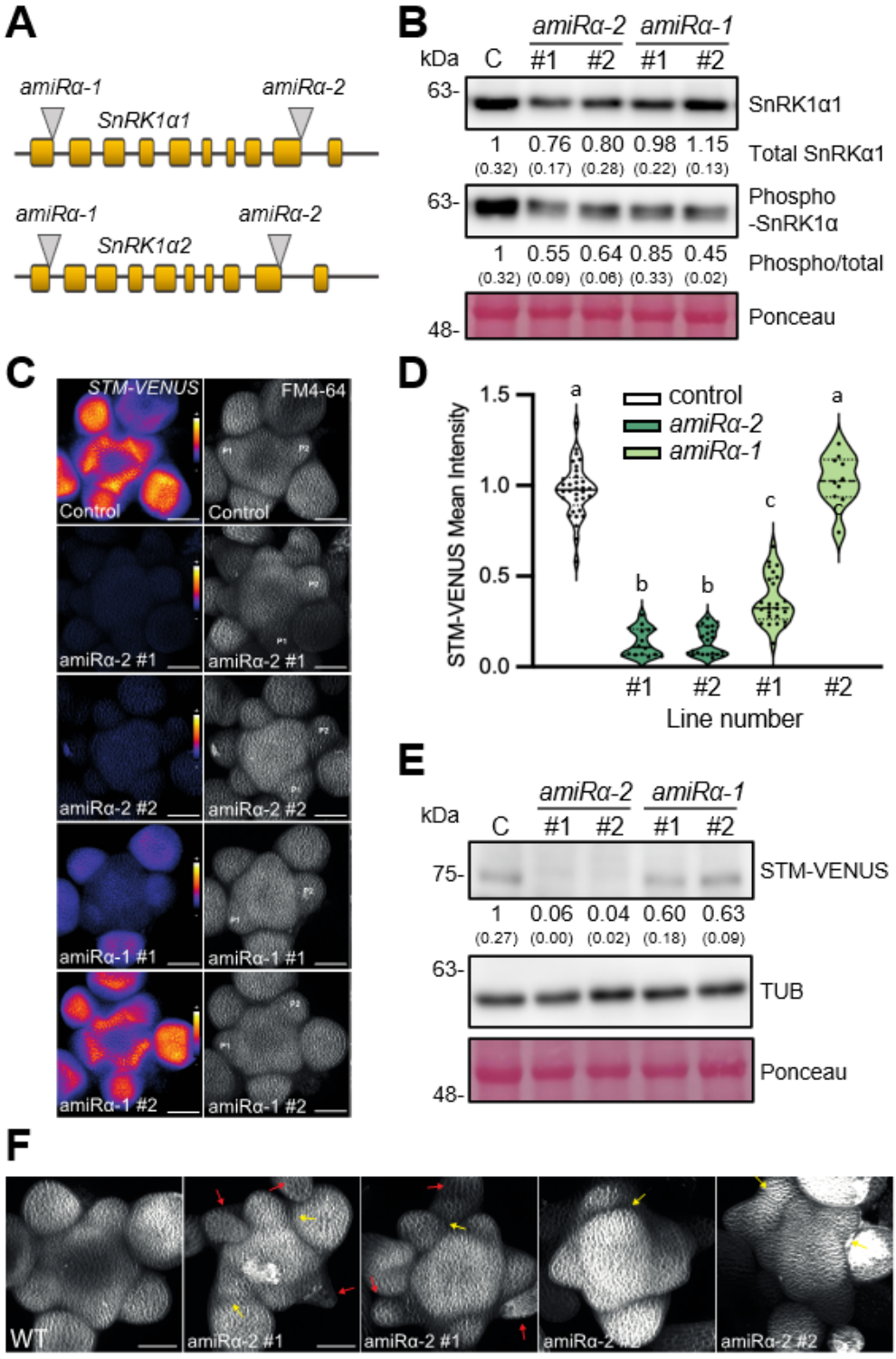
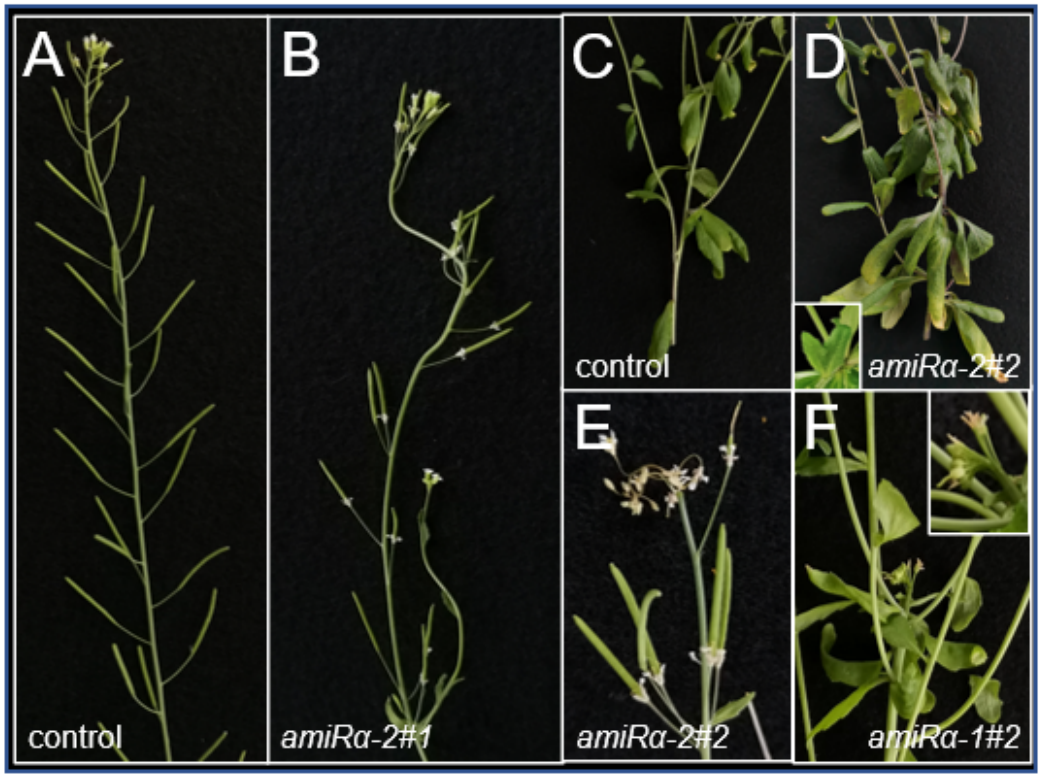

A
amiRα-1
amiRα-2
SnRK1α1
amiRα-1
amiRα-2
SnRK1α2
B
amiRα-2
amiRα-1
kDa
C
#1
#2
#1
#2
63-
SnRK1α1
1 0.76 0.80 0.98 1.15
(0.32) (0.17) (0.28) (0.22) (0.13)
Total SnRKα1
63-
Phospho
-SnRK1α
1 0.55 0.64 0.85 0.45
(0.32) (0.09) (0.06) (0.33) (0.02)
Phospho/total
48-
Ponceau
C
STM-VENUS
FM4-64
Control
Control
amiRα-2 #1
amiRα-2 #1
amiRα-2 #2
amiRα-2 #2
amiRα-1 #1
amiRα-1 #1
amiRα-1 #2
amiRα-1 #2
D
STM-VENUS Mean Intensity
1.5
1.0
0.5
0.0
control
amiRα-2
amiRα-1
a
b
b
c
a
#1
#2
#1
#2
Line number
E
amiRα-2
amiRα-1
kDa
C
#1
#2
#1
#2
75-
STM-VENUS
1 0.06 0.04 0.60 0.63
(0.27) (0.00) (0.02) (0.18) (0.09)
63-
TUB
48-
Ponceau
F
WT
amiRα-2 #1
amiRα-2 #1
amiRα-2 #2
amiRα-2 #2

Figure 4. Silencing *SnRK1α* in the SAM compromises STM expression. A. Schematic localization of *amiRα-1* and *amiRα-2* target sites (grey triangles) in the *SnRK1α1* and *SnRK1α2* transcripts. Yellow blocks correspond to exons. **B.** Immunoblot analyses of SnRK1α T-loop phosphorylation in SAMs of plants expressing *pSTM::amiRα-1* (*amiRα-1*) or *pSTM::amiRα-2* (*amiRα-2*), using antibodies recognizing total SnRK1α1 (upper panel) or SnRK1α1/SnRK1α2 phosphorylated on T175 (phospho-SnRK1α; middle panel). Ponceau staining serves as loading control. Numbers refer to mean SnRK1α1 amounts or mean SnRK1α phosphorylation (phospho-SnRK1α/total SnRK1α1) in the *amiRα* lines relative to the control *STM-VENUS* line (n=2; each a pool of 5 SAMs; in parenthesis, SEM). **C.** Representative meristems expressing *STM-VENUS* together with *pSTM::amiRα-1, pSTM::amiRα-2* or *pSTM::TFP-N7* as a control, and whose membranes were labelled with FM4-64. Left panels show the sum-slice projections of the *STM-VENUS* signal (color-coded using the Fire representation in ImageJ), and the right panels, the sum-slice projection of the FM4-64 signal. Scale bars, 50 μm. *P1* and *P2*, youngest visible and older flower primordia, respectively. **D**. Quantification of the STM-VENUS signal in the SAMs shown in (**C**). Plots show SAM measurements of plants grown as 2 independent batches (except *amiRα-1#2*, which grown as a single batch) normalized by the mean of the control line of each batch (control, n=34; *amiRα-2#1*, n=16; *amiRα-2#2*, n=22; *amiRα-1#1*, n=25; *amiRα-1#2* n=10). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; p<0.05). **E.** Immunoblot analyses of STM-VENUS protein levels in SAMs of the *amiRα* lines as compared to the *STM-VENUS* control using antibodies recognizing STM. TUBULIN (TUB) and Ponceau staining serve as loading controls. Numbers refer to mean STM-VENUS amounts in the *amiRα* lines as compared to the control (n=2; each a pool of 5 SAMs; in

parenthesis, SEM). **F.** Sum-slice projection of WT and *amiRα-2* showing additional defects in meristem organization. Red arrows point at bract-like structures while yellow arrows point at fusions between adjacent floral primordia. Scale bars: 50 µm.

All *amiRα* lines displayed defects in internode elongation, with an increasing frequency of aberrantly long and aberrantly short internodes (Fig. 5A-B, G-H). Defects in internode elongation resulted in clusters of siliques (Fig. 5A,B,E) and what appeared to be aerial rosettes on the main inflorescence (Fig. 5C,D,F; Supplementary Fig. S7A; Table 1). These phenotypes have been linked to reduced *STM* expression and function (Endrizzi et al., 1996; Kanrar et al., 2006; Smith et al., 2004; Ung et al., 2011) and, accordingly, they were more severe in the *amiRα-2* plants*,* where the depletion of STM-VENUS is more pronounced (Fig. 4C-E). Plants expressing *amiRα-2* also exhibited reduced apical dominance with one or two axillary meristems often becoming activated well before flowering (39% and 28% of the *amiRα-2#1,* and *amiRα-2#2* plants, respectively; *n*=18; Supplementary Fig. S7B-C). A less frequent termination of the main meristem was also observed, after which, growth resumed from an axillary meristem (17% of *amiRα-2#1* plants; *n*=18; Supplementary Fig. S7C). Plants expressing *amiRα-1* and *amiRα-2* under the control of the *STM* promoter were also compared to plants harbouring the *pSTM::TFP-N7* construct as a control (both also expressing *pSTM::STM-VENUS*) to ensure that the observed phenotypes were not caused by different copy numbers of *STM* promoters (Fig. 4C,D,F; Supplementary Fig. S7D).

Collectively, these results indicate that SnRK1 plays critical functions in meristem organization and function and that this involves changes in *STM* expression.


A
control
B
amiRα-2#1
C
control
D
amiRα-2#2
E
amiRα-2#2
F
amiRα-1#2

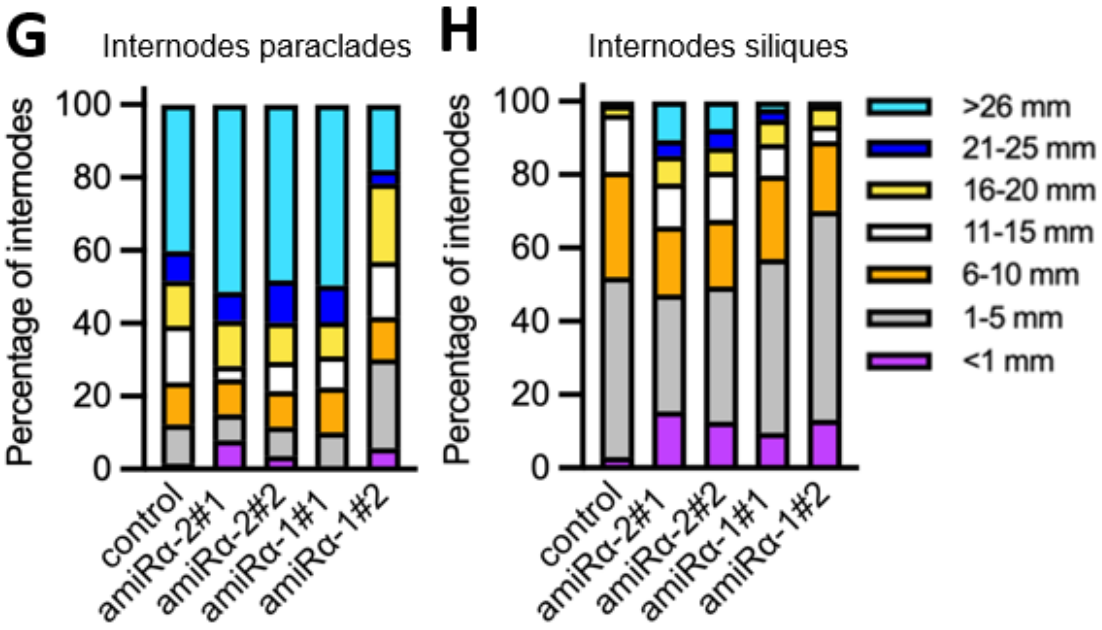

G
Internodes paraclades
Percentage of internodes
100
80
60
40
20
0
control
amiRα-2#1
amiRα-2#2
amiRα-1#1
amiRα-1#2
H
Internodes siliques
Percentage of internodes
100
80
60
40
20
0
control
amiRα-2#1
amiRα-2#2
amiRα-1#1
amiRα-1#2
>26 mm
21-25 mm
16-20 mm
11-15 mm
6-10 mm
1-5 mm
<1 mm

Figure 5. Silencing of *SnRK1α* in the SAM affects meristem function and plant architecture. A-F, Representative images of control (*STM-VENUS*, **A,C**) and *amiRα* (**B,D,E,F**) plants showing irregular internode length (**A,B**), clusters of leaves (**C,D,F**) and siliques (**A,B,E**), and termination of the main inflorescence (**F**) in the *amiRα* lines. Insets show organ fusion between leaves of an aerial rosette (**D**) and between pedicels and the stem (**F**). **G-H**, Quantification of the internode length defects in control and two independent lines of *amiRα-1* and *amiRα-2* mutants. Internode length was determined by measuring the length of the internodes between paraclades (**G**) and between the first 12 siliques (**H**), all counted acropetally. Internode length was scored in the indicated size ranges from the main inflorescence of 18 plants of each genotype. Graphs show the relative frequencies of each size class in the total number of internodes scored. All phenotypes were scored from plants grown under equinoctial conditions until the completion of flowering.

Table I. Number of organs per stem node in *amiRα* plants

Line	Siliques per node	*p*-value	Leaves per node	*p*-value
control	1.4 ± 0.5		1.9 ± 0.8	
amiRα-2#1	2.3 ± 0.8	<0.0001	3.8 ± 1.7	<0.0001
amiRα-2#2	2.2 ± 0.5	<0.0001	3.6 ± 1.5	<0.0001
amiRα-1#1	2.0 ± 0.5	<0.0001	2.8 ± 1.3	<0.0001
amiRα-1#2	1.8 ± 0.7	0.0004	2.1 ± 0.9	>0.9999

Table 1. Quantitative analyses of *amiRα* phenotypes. Measurements were taken from the main inflorescence of 18 plants of the *amiRα* lines and the *STM-VENUS* control line after flowering was completed. Numbers are averages and SD. *p*-values refer to differences between each mutant and the control (Kruskal-Wallis with Dunn´s test).

3.4. Discussion

The capacity to generate organs throughout development is crucial for plant adaptation to the environment, enabling, amongst others, the replacement of leaves lost due to herbivory, the timing of growth to a specific season, or the switch to flower production when conditions are propitious for reproduction. However, how the SAM perceives environmental information and how this is translated into changes in meristem activity are poorly understood.

Here we show that light promotes STM accumulation through sugars. Firstly, a clear correlation between STM-VENUS and sugar levels was observed in the inflorescence apex across different light conditions. STM-VENUS levels were lower in LL-grown or dark-treated plants than in plants grown and maintained under HL (Fig. 1A-C). A similar pattern was observed for sugar accumulation in the inflorescence apex (Fig. 2A), consistent with a previous report on the impact of limiting photosynthetic rates, and thereby sugar supply to the sinks, on the growth and development of reproductive organs and meristem function (Lauxmann et al., 2016). Secondly, STM-VENUS levels declined rapidly when inflorescences were excised from rosettes and this decline was similar in inflorescences maintained in the light or transferred to dark, showing that light is not sufficient to sustain STM levels in this system (Fig. 2B). The reason for this could be that light is sensed in leaves from which a light-related signal travels to the apex and that this remote light sensing and systemic communication is disrupted upon excision of the inflorescence. An alternative explanation is that the signal regulating STM levels is not light itself, but rather photosynthesis-derived sugars, which become rapidly depleted in the excised inflorescence. The fact that the decline in STM-VENUS levels triggered by inflorescence excision could be largely

suppressed by supplementing sucrose in darkness, argues that sucrose is sufficient to sustain STM-VENUS levels and that the effect of light observed in intact plants is indirect *via* photosynthesis and sugar production. The impact of sucrose on STM is in line with the reported effects of nutrients on *WUS* expression and on meristem function. Sugars contribute to meristem activation by inducing *WUS* in young seedlings (Pfeiffer et al., 2016) and nitrogen promotes *WUS* expression and meristem growth in the inflorescence *via* systemic CK signalling (Landrein et al., 2018). However, in contrast to *WUS*, for which transcriptional regulation plays a major role (Landrein et al., 2018; Pfeiffer et al., 2016), we could not detect significant changes in *STM* transcript levels under our different growth conditions or treatments (Fig. 1E and Supplementary Fig. S3), showing that control of protein abundance is an important component of STM regulation. Despite reports linking CK signalling to *STM* expression (Rupp et al., 1999; Teo et al., 2001), STM-VENUS levels did not increase in excised meristems treated with CK (Supplementary Fig. S1D). Even though we did not measure *STM* transcript accumulation under these conditions, this could mean that CK signals may influence STM more indirectly, *e.g.* by affecting *WUS* expression and stem cell number.

The rescue of STM-VENUS levels by sucrose in excised inflorescences suggests that sucrose is sensed locally in the meristem. This is in accordance with the enrichment and activity of the SnRK1 sugar sensor in the SAM (Fig. 3A-D) and with the importance of meristematic SnRK1 for SAM function (Fig. 4 and 5, Supplementary Fig. S6-7). Ubiquitous *SnRK1α1* overexpression caused a reduction in STM-VENUS levels under HL conditions (Fig. 3E) despite the high accumulation of soluble sugars and Tre6P in the rosettes and SAMs of the *SnRK1α-OE* plants (Fig. 3G). Specific silencing of *SnRK1α* in the SAM (using the *STM* promoter), on the other hand, led to a wide range of developmental defects (Fig. 5), demonstrating that SnRK1 acts locally in the

meristem and is crucial for the proper coordination of meristem activities. The severity of the phenotypes caused by SnRK1α depletion contrasts with the apparent lack of developmental defects of the *SnRK1α-OE* line in our growth conditions, consistent with previous studies in which SnRK1α1 overexpression causes mostly developmental delays rather than defective organ development and arrangement (Baena-González & Hanson, 2017; Jamsheer et al., 2021).

The finding that sucrose promotes STM-VENUS expression together with the fact that sugars repress SnRK1 activity may at first sight appear to conflict with the decline in STM-VENUS expression and abnormal meristem function caused by *SnRK1α* silencing. However, despite being generally considered a growth repressor, SnRK1 is also required for cell cycle progression (Guérinier et al., 2013) and for normal growth and development (Baena-González & Lunn, 2020). Indeed, transient *SnRK1α1/α2* downregulation *via* virus-induced gene silencing leads to full growth arrest of plants (Baena-González et al., 2007) and double *snrk1α1 snrk1α2* mutants could thus far not be recovered, suggesting that complete loss of SnRK1α is embryo lethal (Ramon et al., 2019). A similar duality is observed for the AMP-activated protein kinase (AMPK), the homologue of SnRK1 in animals. Despite serving as a brake for cell proliferation through downregulation of TOR activity (González et al., 2020), AMPK is also essential for normal growth and development. For example, complete loss of the AMPKß1 subunit leads to cell cycle defects in neural stem and progenitor cells, causing profound abnormalities in brain development in mice (Dasgupta & Milbrandt, 2009). Along the same lines, hematopoietic stem cell function in mammals is disrupted both upon inactivation and overactivation of TOR signalling, indicating that a fine balance of this central regulator is required for

coordinating proliferation, differentiation, and regeneration (Kalaitzidis et al., 2012).

The phenotypes of the *amiRα* lines are highly reminiscent of those described for partial STM loss-of-function (Clark et al., 1996; Endrizzi et al., 1996; Landrein et al., 2015; Song et al., 2020), including organ fusions, altered phyllotaxy, defective internode elongation with clusters of siliques and leaves and, much less frequently, premature SAM termination (Table 1; Fig. 5; Supplementary Fig. S7). However, whether the impact of sugar signals on STM is direct or indirect through other factors requires further investigation. On one hand, the effect of sucrose and SnRK1α1 overexpression on STM-VENUS suggests that STM regulation occurs at the protein level. The effect of *SnRK1α* silencing, on the other hand, reveals a more complex scenario, involving reduced accumulation of both the STM transcript and protein, and severe developmental abnormalities. The impact on STM could therefore be more indirect and caused by a hormonal imbalance and/or alterations in the cell cycle that feed back to *STM* expression.

Altogether, our work demonstrates that sucrose promotes STM accumulation and that this is counteracted by the SnRK1 sugar sensor, likely to adjust SAM activity to the environment (Fig. 6). Nevertheless, SnRK1 is also essential for meristem integrity, adding to the evidence that SnRK1 performs a dual function in the regulation of growth and that its activity needs to be finely balanced.

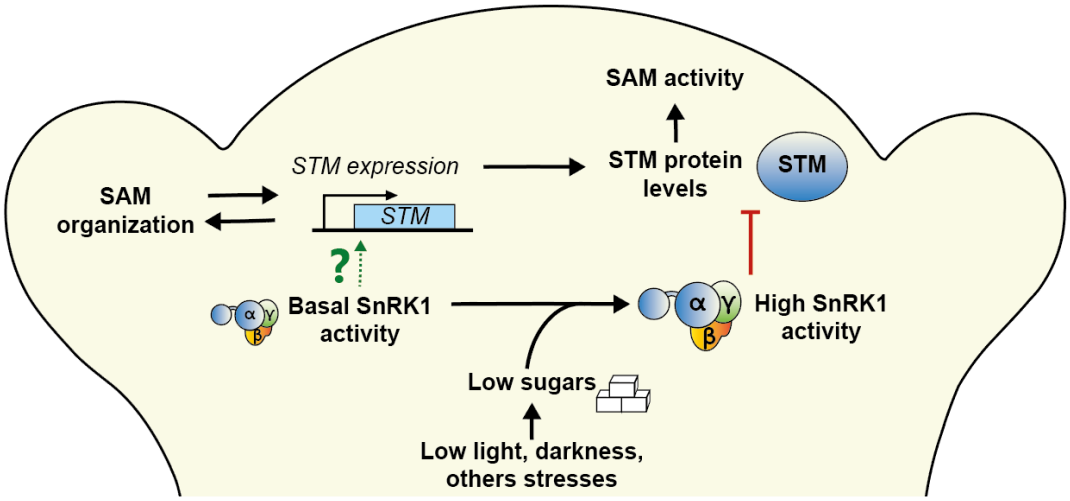


Figure 6. Model for the role of sugars and SnRK1 signalling in the SAM. Under favourable conditions, basal SnRK1 activity is required for meristem organization, with local *SnRK1α* silencing causing reduced *STM* expression and severe phenotypes related to SAM dysfunction. The mechanisms underlying these SnRK1 effects remain unknown (question mark). Under limiting light conditions or other unfavourable situations, sugar levels decrease, leading to a strong activation of SnRK1 signalling. This results in decreased STM protein accumulation, potentially as a means to reduce SAM activity and growth.

3.5. Materials and methods

A list of all primers, antibodies and plant lines used in this study is provided in Supplementary Table 1.

3.5.1. Plant material

All *Arabidopsis thaliana* (L.) Heynh. plants used here are in the Columbia (Col-0) background. The *pSTM::STM-VENUS* line (*STM-VENUS*) was generated by transforming Col-0 plants with the plasmid described by Heisler and colleagues (Heisler et al., 2005; Landrein et al., 2018). The *pTCSn::GFP* was provided by Bruno Müller (Zürcher et al., 2013). The *SnRK1α1-GFP* [*pSnRK1α1::SnRK1α1-GFP::terSnRK1α1/snrk1α1-3*; (Crozet et al., 2016)] and *SnRK1α1-OE* [*35S::SnRK1α1*; (Jossier et al., 2009)] lines were previously described. For expression of STM-VENUS in the *SnRK1α1-OE* background, the *SnRK1α1-OE* and *STM-VENUS* lines were crossed, and homozygous progeny was selected on kanamycin and BASTA. For generating the *pSTM::TFP-N7/STM-VENUS* line, the 5.7 kb promoter of *STM* was amplified by PCR and introduced in a pDONR-P4-P1R plasmid by Gateway recombination cloning (Landrein *et al*, 2015). A triple LR reaction was then performed between this vector, a TFP-N7 recombined with a pDONR221, a Nos-T7 terminator recombined with a pDONR-P2R-P3, and a pH7m34GW destination vector. The resulting gene construct was then introduced into *Arabidopsis* plants already expressing the *STM-VENUS* reporter using *Agrobacterium*. Transformants were selected based on the hygromycin resistance of the pH7m34GW vector. For generating the *pSTM::amiRα* lines, two amiRNAs targeting both *SnRK1α1* and *SnRK1α2* (*amiRα-1* and *amiRα-2*) were designed using the WMD3 Web microRNA designer tool (Schwab et al., 2006) and introduced into the primary *miRNA319a* (At4g23713) backbone in the pHBT95 vector (Martinho et al.,

2015). The resulting primary *miRNA319a*, harbouring the *amiRα-1* or *amiRα-2* sequence, was used to generate the corresponding *amiRα-1* and *amiRα-2* pENTRY 1_2 constructs and recombined with *pSTM* (5.7 kb) pENTRY 4_1r and *NOS-T7*-pENTRY 2r_3 into pK7m34GW (Karimi et al., 2007). The resulting construct was transformed into the *STM-VENUS* line and single-insertion homozygous progeny was selected on kanamycin.

3.5.2. Plant growth conditions

Seeds were sown in excess into individual pots (7 x 7 x 6 cm, W/L/H) with soil (Levington F2) that were thereafter kept in a 4°C room for three days before being transferred to growth cabinets. The pots were covered with transparent plastic lids during the first five days and the plants were thinned out seven days after sowing to leave one plant per pot. For most experiments, seedlings were initially grown in short-day conditions (8 h/16 h light/dark period) for 3-4 weeks and then transferred to continuous light (24 h light, temperature: 22°C) and kept under an irradiance of 60 (LL) or 170 (HL) µmol m^{-2} s^{-1}, provided by white fluorescent lamps. Unless otherwise indicated, plants were grown in HL conditions. For dark treatment, plants grown under HL were put into the dark for 24, 48 or 72 h. For the assays with excised inflorescences and STM-VENUS imaging of *amiRα* lines, seedlings were grown in short-day conditions (8 h/16 h light/dark period) for 3-4 weeks and then transferred to long-day conditions (16 h/8 h light/dark period; 22°C/18°C).

For experiments involving SAM imaging, gene expression or protein analyses, plants were grown in the indicated conditions until bolting, after which SAMs were dissected at the beginning of the flowering stage when the main inflorescence reached 3-5 cm in height.

For phenotyping the *amiRα* lines, seeds were germinated, and plants grown under equinoctial conditions (12 h/12 h light/dark period; 100-110 µmol m^{-2} s^{-1}; 22°C/18°C). Phenotypes were scored when flowering was completed [stage 6.90; (Boyes et al., 2001)].

3.5.3. SAM imaging and quantification

For meristem imaging, the main inflorescence meristem of plants at the beginning of the flowering stage was cut 1-2 cm from the tip, dissected under a binocular stereoscopic microscope to remove all the flowers down to stage 3 [as defined in (Smyth et al., 1990)] and transferred to a box containing Arabidopsis apex culture medium without sucrose (ACM: 2.2 g/l Duchefa Biochemie (www.duchefa-biochemie.com)-MS basal salt mixture with vitamins, pH adjusted to 5.8 with KOH, and 1.6% (w/v) agarose added).

For the time-lapse experiments examining the effect of exogenous CK, meristems were dissected from HL-grown plants and placed in a box of ACM with 1% (w/v) sucrose and 500 nM BAP or the equivalent volume of the BAP solvent (DMSO) as control. Meristems were thereafter returned to the constant HL cabinet for the indicated times and covered with water for imaging.

For the experiments examining the effect of exogenous sugar, inflorescences were dissected at about 3 cm from the apex from HL-grown plants at the beginning of the flowering stage and placed in a 2 mL Eppendorf tube containing 2 mL of liquid ACM (no sucrose) covered with parafilm pierced with a needle so that the inflorescence could be held in air while the base of the stem was submerged in the solution, supplemented with the indicated concentrations of sucrose or sorbitol as osmotic control. Sorbitol and sucrose were used at roughly equivalent concentrations, with 1% sorbitol and

2% sucrose corresponding to 54 mM and 58 mM, respectively. Inflorescences were thereafter kept in darkness inside the growth cabinet for the indicated times. Meristems were then dissected from the excised inflorescences, transferred to a box containing the same sucrose- or sorbitol-supplemented solid (1.6% agarose) medium and covered with water for imaging.

Meristems were imaged in water using a 20X long-distance water-dipping objective mounted either on a LSM880 (Zeiss; www.zeiss.com) or a SP8 (Leica; www.zeiss.com) confocal microscope. Z-stacks of 1-2 µm spacing were taken and the spacing was kept constant within a single experiment.

Confocal Z stacks were analysed using the ImageJ software (https://fiji.sc/) and a custom-made code written in Matlab (Mathworks Inc., Natick, MA). ImageJ was used for generating the panel figures showing the fluorescence reporters in the SAMs. To generate the panels, z-projections (sum slices) of meristems expressing TFP, GFP or VENUS were performed, and the Fire colour code was used to represent the signal. The expression levels of the different fluorescence reporters were analysed by using the Matlab code (see https://gitlab.com/slcu/teamHJ/pau/RegionsAnalysis). *pTCSn::GFP* reporter was analysed by using a previously described pipeline (Landrein et al., 2018), which measures total fluorescence intensity of circular expression domains. STM-VENUS signal and TFP signal were analysed by using a new pipeline that consists on the following. Firstly, a z-sum intensity projection is performed followed by a gaussian blur with a smoothing kernel with standard deviation sigma = 5 µm. Second, a region of interest (SAM core excluding the emerging floral organs) is drawn on the image projection and the mean intensity of the region is extracted. The mean intensity was chosen as a measure of the STM-VENUS and TFP levels instead of the total intensity

to minimize the influence of manually drawing the region of interest and the new buds on the signal.

To measure the correlation between the folding of the boundary and the size of the primordia in *pSTM::amiRα-1* and *amiRα-2* lines, maximal projections were performed to outline the stage-2 primordia (Smyth et al., 1990) and measure their projected area. Longitudinal sections passing through the middle of the primordia were also performed to measure the folding of the boundary using the angle tool of ImageJ as previously described (Landrein et al., 2015). The relationship between primordia size and the folding of the boundary appeared to be more linear for smaller primordia. Therefore, only stage-2 primordia up to 4000 µm² in size were considered for these measurements.

3.5.4. Protein extraction, quantification, and immunoblotting

For extraction of total protein for the immunoblot analyses, one leaf or complete inflorescence meristems (five per replicate) were ground in liquid N_2. Finely ground tissue (approximately 30-40 mg per sample) was extracted in 1.5 volumes of buffer [150 mM NaCl, 1% (v/v) Triton-X, 50 mM Tris-HCl (pH 8.0), 3 mM DTT, supplemented with 50 µM MG132, 1:20 Complete EDTA-free Protease Inhibitor Cocktail (Roche), and 1:500 Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich; www.sigmaaldrich.com)]. Homogenates were cleared by centrifugation at 21130 x g for 15 minutes at 4°C, and supernatants were used for total protein quantification (Pierce 660 nm protein assay reagent; www.thermofisher.com). Single use aliquots of 40 µg total protein were prepared and denatured in Laemmli buffer (Laemmli, 1970) before storage at -20°C. Total protein samples were separated by SDS-PAGE in 10% acrylamide gels, transferred to PVDF membranes (wet transfer at 4°C,

100 V, 70 min) and probed using 1:1000 dilutions of STM, p-AMPK, SnRK1α1 or tubulin antibodies.

Chemiluminescent detection was performed using a 1:10,000 dilution of Peroxidase AffiniPure goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch; www.jacksoniummuno.com), and SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Band intensity was quantified using ImageJ. The bands of interest were selected with the rectangle tool and the corresponding intensity peaks were plotted. The peaks were separated using the line tool after which their area was measured. For quantifying STM-VENUS and SnRK1α1 amounts, band intensity values were normalized to the values corresponding to their respective Rubisco band in the Ponceau-stained gel. For quantifying SnRK1α phosphorylation, band intensity values of the phospho-SnRK1α immunoblot were normalized to the values corresponding to their respective SnRK1α1 immunoblot.

3.5.5. RNA extraction, cDNA synthesis and qRT-PCR

Total RNA was extracted from approximately 30-40 mg of finely ground tissue, with the Plant Nucleospin RNA extraction kit (Macherey-Nagel; www.mn-net.com), according to the manufacturer's instructions. DNAse-treated RNA (1 µg) was used for synthesis of cDNA libraries, using SuperScript III Reverse Transcriptase (Life Technologies;www.thermofisher.com).

qRT-PCR analyses were performed in 384-well reaction plates using the QuantStudio™ 7 Flex Real- Time PCR System (Thermo Fisher). The reactions were prepared in a total volume of 10 µL containing 1 µL of cDNA (diluted 1:10) corresponding to 2.5 ng of RNA, 5 µL of iTaq Universal SYBR

Green Supermix (BioRad www.bio-rad.com) and 0.8 µL of each gene-specific 5 µM primer. No-template and -RT controls were included for each gene in comparative gene expression analyses.

The $2^{-\Delta\Delta Ct}$ method was used for relative quantification. Expression values were normalised to the geometric mean of Ct values obtained for the following reference genes: *UBQ10* (At4g05320) and *UBC21* (At5g25760). Expression of reference genes, *STM* (At1g62360), *HB25* (At5g65410), *AIL7* (At5g65510), *DIN10* (At5g20250), *DRM2* (At2g33830) and *SEN5* (At3g15450) was assessed using the primers described in Table S1.

3.5.6. Sugar measurements

Soluble sugars were extracted from aliquots (15-20 mg) of frozen tissue powder using chloroform-methanol as described in (Lunn et al., 2006). Sucrose, glucose and fructose were quantified by high-performance hydrophilic interaction chromatography on a 150 x 2.1 mm Luna Omega Sugar (Phenomenex Inc.; Aschaffenburg, Germany; www.phenomenex.com) column, fitted with a 4 x 2.0 mm Security Guard Cartridge (Phenomenex) and coupled to a QTrap 5500 triple quadrupole mass spectrometer (AB Sciex, Foster City, USA; sciex.com). Samples were spiked with ^{13}C-labelled internal standards for correction of ion suppression and other matrix effects (Feil & Lunn, 2018). The column was maintained at 25°C and equilibrated with 90 % (v/v) acetonitrile, 5 % (v/v) isopropanol, 5 % (v/v) water (eluent A) before injection of samples (1 µl). Sugars were eluted (flow rate 0.25 ml min-1) with the following gradient, obtained by mixing eluent A with eluent B [5 % (v/v) acetonitrile, 25 % (v/v) isopropanol, 70 % (v/v) water): 0 – 2.5 min [10 % B]; 2.5 - 25 min [10 - 30 % B]; 25 – 30.5 min [30 % B]; 30.5 – 31 min [30 – 10 % B]; 31 – 40 min [10 % B]. Sugars were quantified by tandem mass

spectrometry using the parent/product ion transitions as described in (Feil & Lunn, 2018). Tre6P was quantified in chloroform-methanol extracts as described in (Lunn et al., 2006) with modifications as described in (Figueroa et al., 2016).

3.6. Acknowledgements

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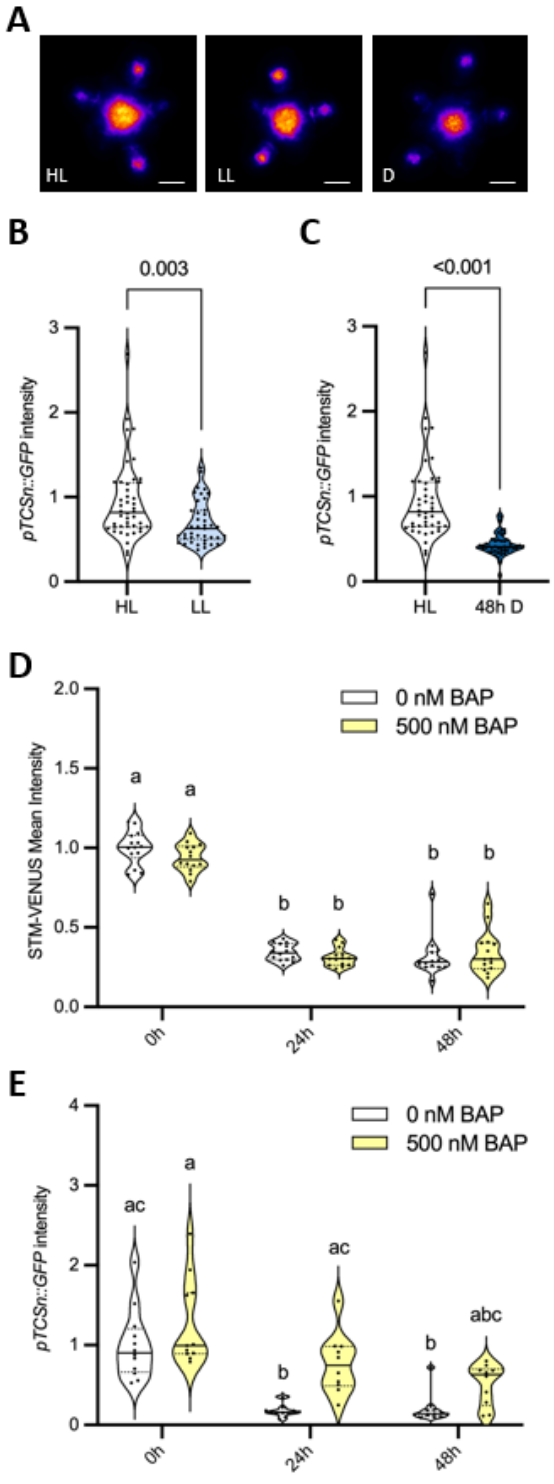
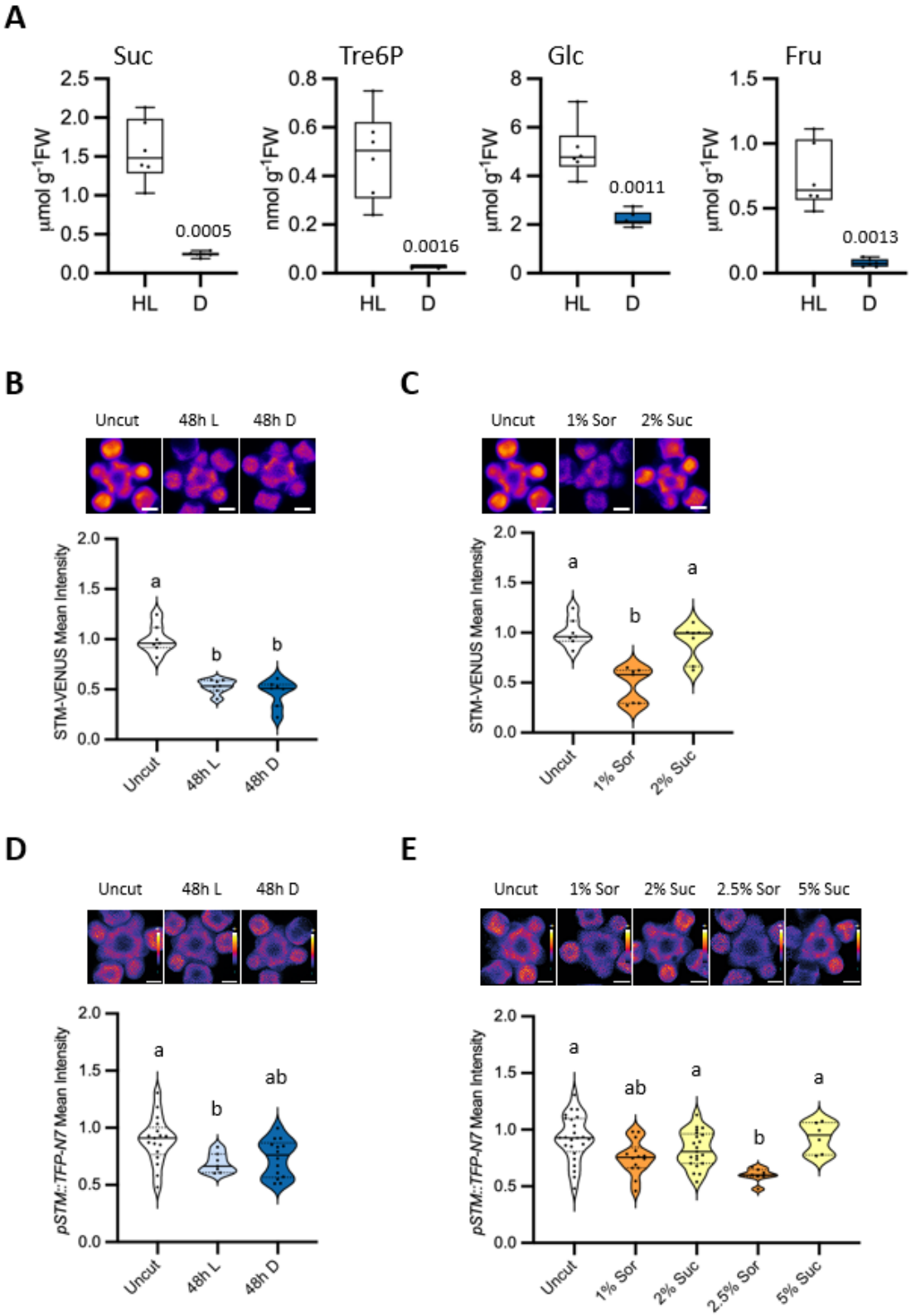
3.7. Supplementary figures

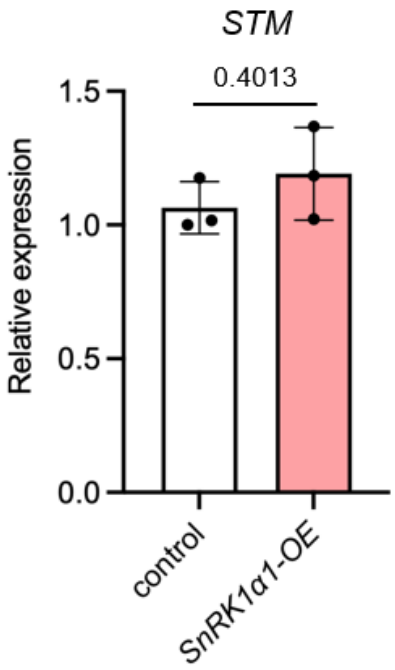


Supplementary Figure S1. Effect of cytokinin on STM levels. A, Representative GFP images from *pTCSn::GFP* SAMs from plants grown under high light (HL; 170 µmol m^{-2} s^{-1}) or low light (LL; 60 µmol m^{-2} s^{-1}) conditions and of plants transferred from HL to darkness (D) for 48 h. Scale bar, 50 µm. **B,** GFP quantification from SAMs of *pTCSn::GFP* plants grown under HL or LL conditions. Plots show SAM measurements of plants grown as 3 independent batches normalized by the mean of the HL condition of each batch (HL: *n*=44, LL: *n*=41). Student's *t*-test (*p*-values shown). **C,** GFP quantification from SAMs of *pTCSn::GFP* plants grown in HL and transferred to D or kept under HL for 48 h. Plots show SAM measurements of plants grown as 3 independent batches normalized by the mean of the HL condition of each batch (HL, *n*=44; 48 h D, *n*=45). Student's *t*-test (*p*-values shown). **D,** Effect of cytokinin (BAP) application on the activity of the *STM-VENUS* reporter. SAMs of plants grown under HL conditions were excised and placed in medium supplemented or not with 500 nM BAP for the indicated times. Plots show SAM measurements of plants grown as 2 independent batches normalized by the mean of the uncut (0h) condition of each batch (*n*=14 for each of the indicated conditions). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; $p<0.05$). **E,** Effect of cytokinin (BAP) application on the activity of the *pTCSn::GFP* reporter. SAMs of plants grown under HL conditions were excised and placed in medium supplemented or not with 500 nM BAP for the indicated times. Plots show SAM measurements of plants grown as 2 independent batches normalized by the mean of the uncut (0h) condition of each batch (all 0 nM BAP conditions, *n*=12; 0h 500 nM BAP, *n*=12; 24 h 500 nM BAP, *n*=10; 48 h 500 nM BAP, *n*=10). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; $p<0.05$).

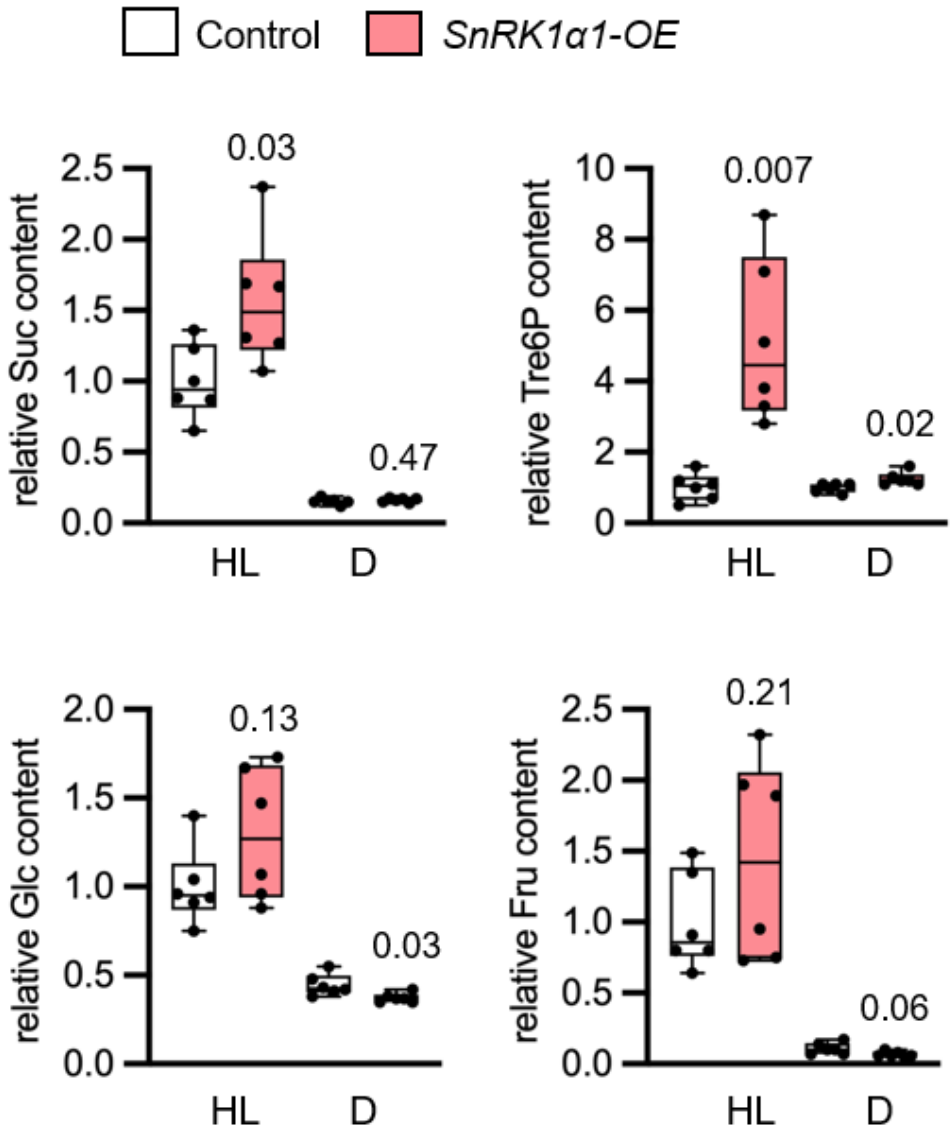

A
Suc
Tre6P
Glc
Fru
µmol g-1FW
nmol g-1FW
0.0005
0.0016
0.0011
0.0013
HL
D
B
Uncut
48h L
48h D
STM-VENUS Mean Intensity
C
1% Sor
2% Suc
D
pSTM::TFP-N7 Mean Intensity
E
2.5% Sor
5% Suc

Supplementary Figure S2. Effect of sugar on *STM* promoter activity. A, Effect of light on the levels of soluble sugars in rosettes of *pSTM::STM-VENUS* plants grown in high light (HL) and transferred to darkness (D) or kept in HL for 48 h. Suc, sucrose; Tre6P, trehalose 6-phosphate; Glc, glucose; Fru, fructose. Plots show measurements of 6 whole rosettes from plants grown as one batch. Welch´s *t*-test (*p*-value shown). **B,** Effect of light on STM-VENUS levels in cut inflorescences. Inflorescences of *pSTM::STM-VENUS* plants grown under HL were cut and placed in medium without sugar for 48 h under HL (L) or dark (D) conditions, after which the SAMs were dissected and imaged (VENUS). Upper panel, representative STM-VENUS images of SAMs. Scale bar, 50 µm. Lower panel, plots showing SAM measurements of plants grown as one batch normalized by the mean of the uncut condition (uncut, n=7; 48 h L, n=7; 48 h D, n=7). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; p<0.05). **C,** Effect of sugar on STM-VENUS levels in cut inflorescences. Inflorescences of *pSTM::STM-VENUS* plants grown under HL condition were cut and placed under darkness for 48 h in medium with 1% sucrose or 2% sorbitol as osmotic control. SAMs were thereafter dissected and imaged (VENUS). Upper panel, representative STM-VENUS images of SAMs. Scale bar, 50 µm. Lower panel, plots showing SAM measurements of plants grown as one batch normalized by the mean of the uncut condition (uncut, n=7; 1% Sor, n=7; 2% Suc, n=7). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; p<0.05). The same batches of HL-grown uncut plants served as controls for the experiments shown in (**B)** and (**C)**. **D,** Effect of light on *STM* promoter activity in cut inflorescences. Inflorescences of *pSTM::STM-VENUS/pSTM::TFP-N7* plants grown under HL were treated as in (B) and dissected SAMs were imaged (TFP). Upper panel, representative *pSTM::TFP-N7* images of SAMs. Scale bar, 50 µm. Lower panel, plots

showing SAM measurements of plants grown as 2 independent batches (except 48h L samples, which generated from a single batch) normalized by the mean of the *uncut* condition of each batch (uncut, *n*=17; 48 h L, *n*=7; 48 h D, *n*=14). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; $p<0.05$). **E,** Effect of sugar on *STM* promoter activity in cut inflorescences. Inflorescences of *pSTM::STM-VENUS;pSTM::TFP-N7* plants grown under HL were cut and placed under darkness for 48 h in medium with sucrose (Suc; 2% and 5%) or sorbitol (Sor; 1% and 2.5%) as osmotic control. SAMs were thereafter dissected and imaged (TFP). Upper panel, representative *pSTM::TFP-N7* images of SAMs. Scale bar, 50 µm. Lower panel, plots showing SAM measurements of plants grown as 2-3 independent batches (except 2.5% Sor and 5% Suc, which were grown as a single batch) normalized by the mean of the *uncut* condition of each batch (uncut, *n*=24; 1% Sor, *n*=14; 2% Suc, *n*=21; 2.5% Sor, *n*=7; 5% Suc, *n*=6). Different letters indicate statistically significant differences (Kruskal-Wallis with Dunn´s test; $p<0.05$).

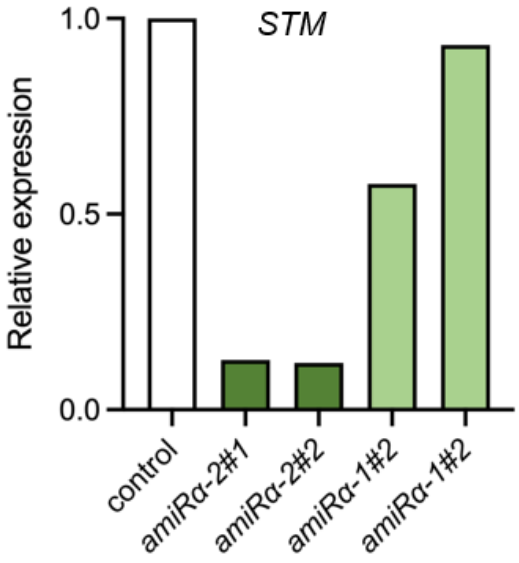


Supplementary Figure S3. Effect of ubiquitous SnRK1α1 overexpression on *STM* levels. RT-qPCR analyses of *STM* in SAMs of control and *SnRK1α1-OE* plants grown under high light conditions (170 µmol m^{-2} s^{-1}). Graphs correspond to the average of 3 independent samples, each consisting of a pool of 5 SAMs. Paired ratio *t*-test (*p*-value shown).

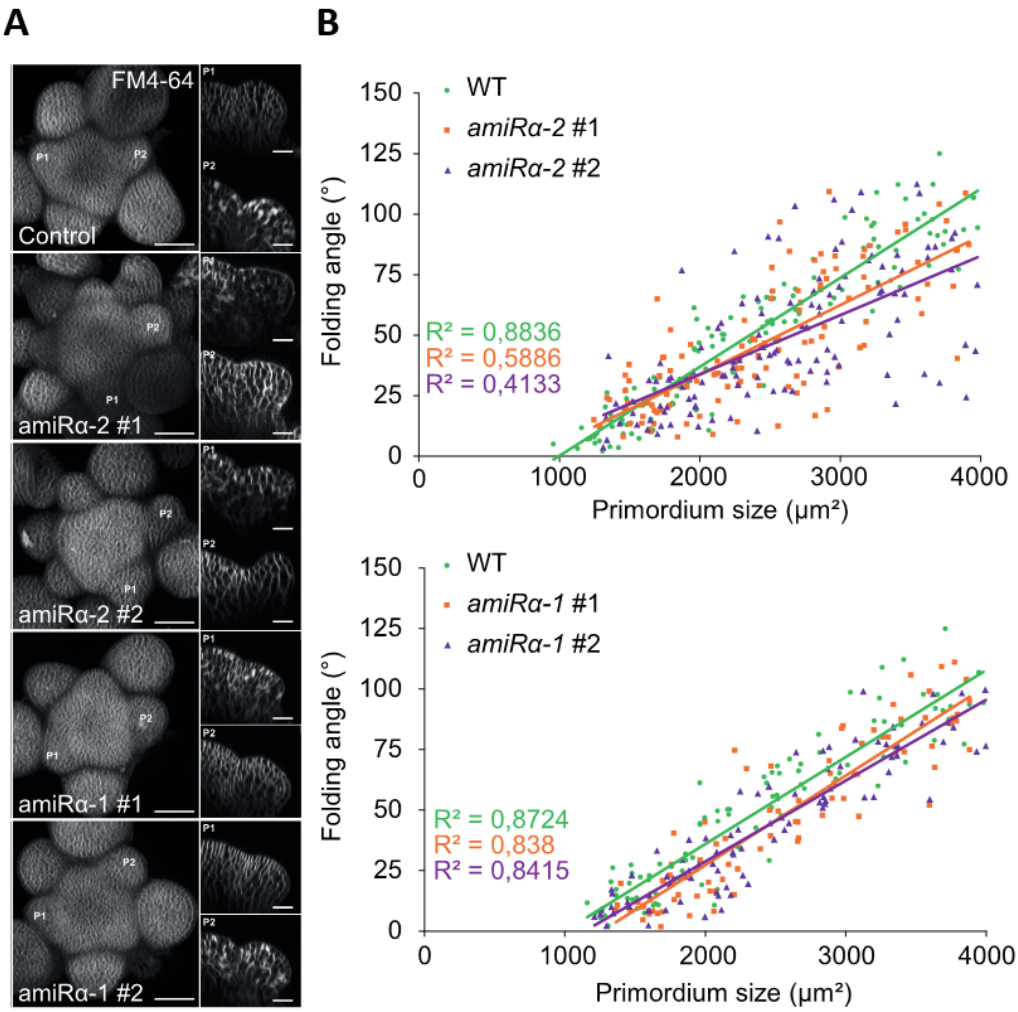


Supplementary Figure S4. Effect of ubiquitous SnRK1α1 overexpression on the accumulation of soluble sugars. Control and *SnRK1α1-OE* plants were grown under HL conditions and transferred to darkness (D) or kept under HL for 48 h. Plots show measurements of 6 whole rosettes from plants grown as one batch, with *SnRK1α1-OE* values expressed in comparison to the

control. Suc, sucrose; Tre6P, trehalose 6-phosphate. Glc, glucose; Fru, fructose. Welch´s *t*-test (mutant *vs*. control for each condition; *p*-value shown).

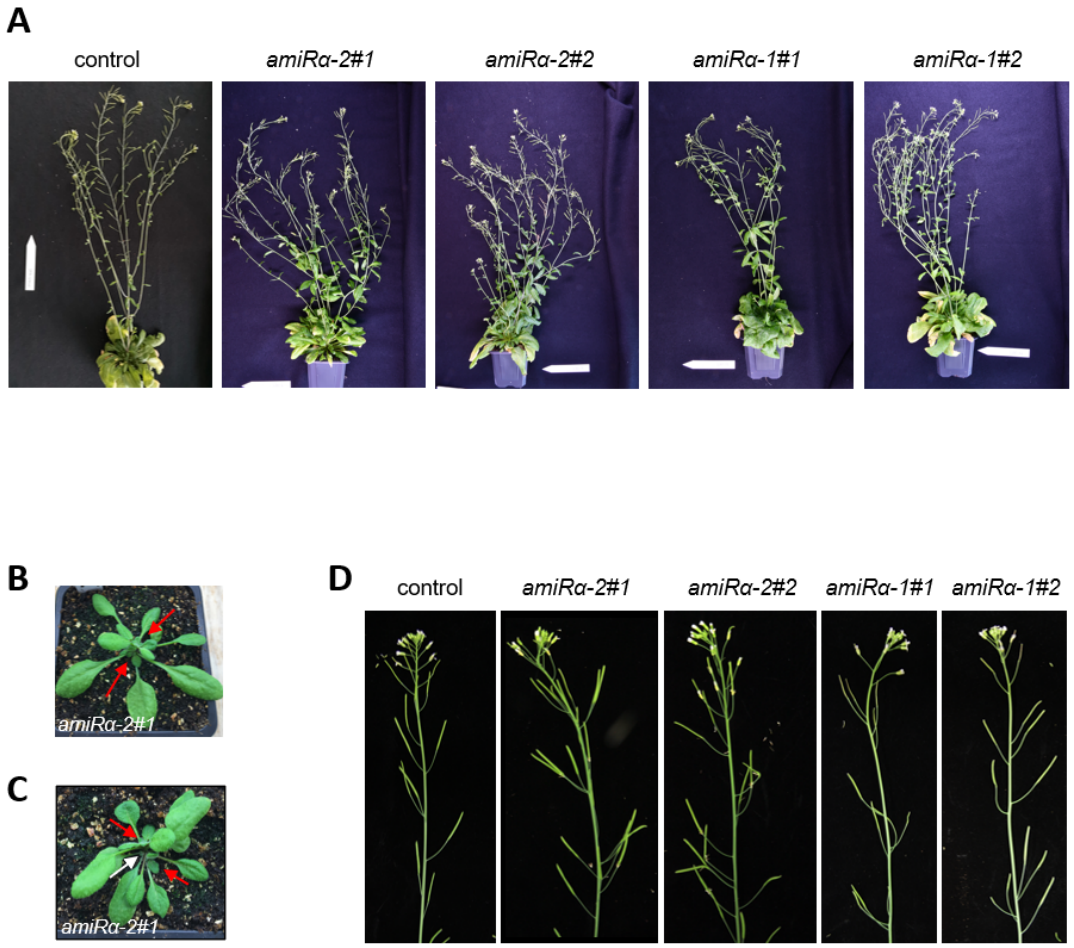


Supplementary Figure S5. Effect of SnRK1α depletion in the SAM on *STM* levels. RT-qPCR analyses of *STM* in SAMs of control and *pSTM::amiRα* plants grown under high light conditions (170 µmol m^{-2} s^{-1}). Two independent lines of *pSTM::amiRα-2* (*amiRα-2#1* and *amiRα-2#2)* and *pSTM::amiRα-1* (*amiRα-1#1* and *amiRα-1#2*) were used. Graphs correspond to a pool of 5 SAMs.



Supplementary Figure S6. Effect of SnRK1α depletion in the SAM on organ boundary formation. A, Representative meristems expressing *STM-VENUS* together with *pSTM::amiRα-1, pSTM::amiRα-2* or *pSTM::TFP-N7* as a control, and whose membranes were labelled with FM4-64. Left panels show the sum-slice projection of the FM4-64 signal and the right panels, two longitudinal sections showing the SAM-organ boundary of two stage-2 primordia. Note the reduced folding of the boundary and reduced bulging of the primordia in the *pSTM::amiRα-2* line. Scale bars, projections: 50 µm, sections: 20 µm. **B,** Folding angle of the boundary as a function of the size of

the primordium in control plants (expressing *pSTM::TFP-N7*) as compared to *amiRα-2* (upper graph) or *amiRα-1* plants (lower graph). Plants in the *amiRα-2* experiment were grown as 3 independent batches. Control, *n*=126 primordia from 29 SAMs; *amiRα-2*#1, *n*=121 primordia from 25 SAMs; *amiRα-2*#2, *n*=120 primordia from 26 SAMs). Plants in the *amiRα-1* experiment were grown as 2 independent batches. Control, *n*=95 primordia from 22 SAMs; *amiRα-1*#1, *n*=84 primordia from 22 SAMs; *amiRα-1*#2, *n*=87 primordia from 21 SAMs. Data were fitted using a linear regression (see Methods).



Supplementary Figure S7. Silencing of *SnRK1α* in the SAM compromises meristem function and plant architecture. A,

Representative images of control plants and two independent *amiRα-2* and *amiRα-1* lines grown under equinoctial conditions until the completion of flowering. **B-C**, Representative images of 22d-old plants of the most severely affected *amiRα* line (*amiRα-2*#1) showing activation of axillary meristems before flowering (red arrows in **B-C**) and termination of the main meristem (white arrow in **C**). **D,** Representative images of control plants (expressing *pSTM::TFP-N7*) and two independent *amiRα-2* and *amiRα-1* lines grown under short-day conditions for 3 weeks and then transferred to long-day conditions until the completion of flowering.

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CHAPTER IV

Molecular link between SnRK1 and the SHOOT MERISTEMLESS

Author contributions: Filipa Lopes and Elena Baena-González conceived the research plan and designed the experiments. Filipa Lopes performed the experiments. Filipa Lopes and Elena Baena-González analysed and interpreted data.

4.1. Abstract

In plants, stem cells reside in special niches called meristems. The shoot apical meristem (SAM) is responsible for keeping a balance between cell proliferation and differentiation in the aerial part of the plant, ensuring the production of organs throughout the entire life cycle.

In *Arabidopsis*, the gene *SHOOT MERISTEMLESS (STM)*, a KNOTTED1-like homeobox (KNOX) gene, is required for meristematic identity and is essential both for the establishment and maintenance of the SAM. The nuclear localization, DNA binding affinity, and activity of STM requires the interaction with BEL1-like (BELL) proteins, such as BEL1-like BEL1-LIKE HOMEODOMAIN 8 (BLH8), BLH9, and ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1).

Our previous work showed that light promotes the accumulation of the STM protein and that this is largely dependent on photosynthesis-derived sugars and inhibited by the Sucrose Non-Fermenting 1 (SNF1)-related kinase1 (SnRK1), a major regulator of growth under nutrient-limiting conditions. However, it remains to be addressed how sugar signals affect STM function and hence SAM activity.

Here, we further explore the molecular connection between the SnRK1 energy sensor and STM function, showing that the SnRK1α1 catalytic subunit interacts with several key transcription factors that regulate SAM function, namely STM, BLH8, BLH9, ATH1, and WUSCHEL (WUS). We demonstrate that SnRK1α1 imposes a strong repressive effect on STM activity that could involve direct phosphorylation of STM, or its partner transcription factors.

4.2. Introduction

Unlike most animals, plants produce new organs such as leaves, stems, and flowers, throughout their entire life cycle due to the activity of stem cell reservoirs known as meristems. To enable continuous organ production, plants have developed strategies to balance stem cell proliferation and differentiation. This ensures a tight coordination between the differentiation of stem cells into new organ primordia and their self-renewal through slow divisions for replenishment of the stem cell pool.

The establishment and sustained function of the shoot apical meristem (SAM) requires the activity of several transcriptional networks, hormone signalling pathways, and the trafficking of transcriptional regulators. In Arabidopsis, a well-known mechanism for SAM maintenance relies on a negative feedback loop between the CLAVATA3 (CLV3) peptide and the WUSCHEL (WUS) transcription factor (TF) (Brand et al., 2000; Schoof et al., 2000) Chapter II). WUS promotes stem cell identity by repressing stem cell differentiation and directly induces *CLV3* expression. In turn, CLV3 signalling negatively regulates *WUS* transcription. Hence, this negative feedback loop tightly modulates the size of the stem cell pool (Chapter II).

Additional key regulators of SAM activity include the three-amino acid length extension (TALE) superclass of homeobox TFs, such as KNOTTED1-like (KNAT or KNOX) and BELL-like (BLH or BELL), which are both members of the homeobox TF family (Hay & Tsiantis, 2010; Hepworth & Pautot, 2015).

SHOOT MERISTEMLESS (STM), a class I KNOX TF, is essential for the establishment and maintenance of the SAM (Barton & Poethig, 1994; Long et al., 1996). In plants harbouring strong *stm* alleles, such as *stm-1*, loss of STM function leads to the lack of a functional SAM, causing growth arrest at the seedling stage and the fusion of the cotyledon petioles (Barton & Poethig,

1994). In contrast, weak *stm* mutants form a SAM that initiates primordia shortly after germination, but the stem cell niche is eventually depleted due to stem cell differentiation and incorporation into organ primordia. Additionally, multiple other defects have been reported to be associated with reduced *STM* expression, such as defects in floral organ development and internode elongation (Barton & Poethig, 1994; Clark et al., 1996; Endrizzi, Karin K. et al., 1996; Long et al., 1996; Song et al., 2020).

STM is expressed very early during embryogenesis, at the globular-heart stage, and its expression remains in the meristem throughout shoot development but is downregulated in organ primordia (Hay & Tsiantis, 2010; Long et al., 1996). Downregulation of *STM* expression in this region, together with other signals, such as high auxin levels, act as trigger for the formation of organ primordia (Benková et al., 2003; Heisler et al., 2005; Reinhardt, 2000).

STM also coordinates the accumulation of gibberellin (GA) and cytokinin (CK), that play important antagonistic roles in SAM activity (Jasinski et al., 2005; Yanai et al., 2005). GAs drive cell growth and differentiation in the developing organ primordia (Olszewski et al., 2002). In turn, STM promotes stem cell identity partly by repressing GA accumulation in the SAM (Hay et al., 2002; Jasinski et al., 2005).

In contrast, CKs are required for the maintenance of stem cells in an undifferentiated state and for their proliferation, notably by promoting *STM* expression (Rupp et al., 1999). In turn, STM promotes CK accumulation by activating the CK biosynthesis gene *ISOPENTENYL TRANSFERASE7* (*IPT7*) (Jasinski et al., 2005; Yanai et al., 2005). Application of exogenous CK or targeted induction of *IPT7* expression in the SAM could partially restore meristem activity in *stm* mutants (Jasinski et al., 2005; Yanai et al., 2005). However, CK could not fully replace STM function in SAM formation and

maintenance, supporting the idea that STM also plays CK-independent roles in meristem function (Scofield et al., 2014, 2013).

In addition to promoting stem cell identity, STM plays a role in boundary specification, which is required for proper organ separation (Scofield et al., 2018, Landrein et al, 2015). Boundary formation in the shoot organs is mainly regulated through the TFs CUP-SHAPED COTYLEDON 1 (CUC1), CUC2 and CUC3, as demonstrated by the organ fusions observed in *cuc1* and *cuc2* loss-of-function mutants (Aida et al. 1997, 1999). During embryogenesis, *CUC* genes are initially expressed throughout the SAM and their expression is required for the activation of *STM* (Aida et al., 1999; Long et al., 1996), which subsequently limits *CUC* expression to the boundaries of the cotyledon primordia (Takada et al., 2001). Later in plant development, STM binds to the *CUC1* promoter and directly promotes its expression (Spinelli et al., 2011). However, STM also promotes the expression of *CUC1*- and *CUC2*-targeting microRNAs of the miR164 family, and thus act both as a positive and a negative regulator of CUC factors (Scofield et al., 2018; Spinelli et al., 2011).

The activity of key regulators of SAM maintenance and function is highly dependent on their spatial regulation (Lenhard, 2003; Schlegel et al., 2021). The functions of STM in development are no exception and depend on the cell-to-cell movement of both STM protein and RNA (Balkunde et al., 2017; Liu et al., 2018; Xu et al., 2011). The nuclear localization of STM depends on its heterodimerization with BELL proteins (Cole et al., 2006; Rutjens et al., 2009). Interestingly, the conserved BELL domain is sufficient for the interaction with STM, but not for the nuclear import of STM protein (Cole et al., 2006). The interaction with the BELLs is also essential for the DNA binding affinity of STM (Bellaoui et al., 2001; Kim et al., 2013; Muller et al., 2001; Smith et al., 2002). In complement, STM may also play a role in controlling the subcellular localization of the BELLs by competing with the nuclear export

machinery for binding the two nuclear export signal (NES) sequences that reside within the BELL domain (Rutjens et al., 2009).

From the 13 members of the BELL family, BLH9 [also known as PENNYWISE (PNY), BELLRINGER, REPLUMLESS, LARSON or VAAMANA], POUNDFOOLISH (PNF)/BLH8, and ARABIDOPSIS THALIANA HOMEOBOX GENE1 (ATH1), are the best characterized and were shown to interact with STM *in vitro* and *in vivo* (Bellaoui et al., 2001; Bhatt et al., 2004; Byrne et al., 2003; Cole et al., 2006; Muller et al., 2001; Rutjens et al., 2009; Smith et al., 2002; Smith & Hake, 2003). These three TFs are known to be involved in multiple developmental processes, ranging from defining the spatial expression pattern of boundary genes, to the control of floral transition and inflorescence patterning (Andrés et al., 2015; Bhatt et al., 2004; Byrne et al., 2003; Gomez-Mena & Sablowski, 2008; Kanrar et al., 2008; Proveniers et al., 2007; Roeder et al., 2003; Rutjens et al., 2009; Smith & Hake, 2003). Both PNY and PNF are required for proper STM activity, and the *pny* mutation was shown to enhance *stm* mutant phenotypes (Bhatt et al., 2004; Byrne et al., 2003; Kanrar et al., 2006; Smith et al., 2004). Nevertheless, the phenotype of the *pny pnf* mutant is distinguishable from that of *stm*, suggesting that PNY and PNF are involved in developmental processes that are not fully shared with STM (Hake et al., 2004; Kanrar et al., 2006, 2008; Ung et al., 2011).

Besides the BELLs, STM was also recently shown to interact with WUS to modulate stem cell identity. STM promotes *CLV3* expression through direct binding to its promoter, thereby facilitating the binding of WUS to this site through a WUS-STM interaction (Su et al., 2020). Interestingly, *STM* expression is also activated by WUS, as a mechanism to reinforce WUS-mediated stem cell activity.

In addition to internal signals, several environmental cues can influence meristem function through hormone signalling and the CLV3–WUS loop (Shi

and Vernoux, 2022, Chapter II). Light and metabolic signals were shown to converge on the TARGET OF RAPAMYCIN (TOR) pathway to regulate the activity of vegetative meristems. The activation of TOR in response to light and glucose signals leads to phosphorylation of the E2Fa TF and thereby to increased expression of S-phase genes and increased cell proliferation (Li et al., 2017; Xiong et al., 2013). Moreover, TOR activity is required for the positive effect of light and sugars on *WUS* expression, and this is at least partly dependent on the activity of two CK degrading enzymes from the CYTOKININ OXIDASE (CKX) family (Janocha et al., 2021; Pfeiffer et al., 2016).

Sugars serve as key building blocks as well as important signalling molecules reflecting the plant energy status and, therefore, the ability to sense sugar levels and maintain an energy balance is key to adaptation and survival (Lastdrager et al., 2014; Wingler, 2017). The signalling pathways centred on the conserved TOR and SnRK1 kinases lie at the heart of energy sensing and play crucial and antagonistic roles in translating this information into metabolic and developmental adaptations (Baena-González & Hanson, 2017; Jamsheer K et al., 2021). TOR promotes protein synthesis and rRNA expression in nutrient favourable conditions. By contrast, SnRK1 is a major regulator of growth under nutrient-limiting conditions. This kinase is pivotal for the adaptation of cellular metabolism to energy-deficiency situations, shifting cellular metabolism from an anabolic towards a catabolic mode (Baena-González & Hanson, 2017; Jamsheer K et al., 2021)

Our previous work showed that light promotes STM protein accumulation and that this process is dependent on photosynthesis-derived sugars and inhibited by the SnRK1 kinase (Chapter III). Furthermore, local silencing of SnRK1 subunits in the SAM caused phenotypes highly reminiscent of those described for partial *STM* loss-of-function mutants (Clark et al., 1996; Endrizzi,

Karin K. et al., 1996; Landrein et al., 2015; Song et al., 2020), including organ fusions, altered phyllotaxis, defective internode elongation with clusters of siliques and leaves and, much less frequently, premature SAM termination (Chapter III). However, the mechanisms linking sugar signalling through SnRK1 to SAM function, and thus, STM regulation, are still to be unveiled.

Here, we further investigate the molecular connection between the SnRK1 kinase and STM function. Our results show that the SnRK1α1 catalytic subunit interacts physically with several key regulators of SAM activity, such as STM and its partner transcription factors BLH8, BLH9, ATH1, and WUS, and that this interaction strongly impinges on STM activity, potentially *via* phosphorylation.

4.3. Results

4.3.1. SnRK1 interacts with major SAM regulators

As a first step to test whether the reported impact of SnRK1 in the SAM (Chapter III) is due to a direct action of the kinase on major meristem regulators, we performed pairwise yeast two-hybrid (Y2H) assays (Fig. 1A). The coding sequence (CDS) of the SnRK1 catalytic subunit SnRK1α1, fused with the GAL4 DNA-binding domain (BD), was co-transformed with constructs harbouring the full-length CDS of the TFs STM, WUS, BLH8, BLH9 and ATH1 fused to the GAL4 activation domain (AD). As controls, pGBKT7-SnRK1α1 and pGADT7-TF constructs were also co-transformed with the respective complementary empty vectors. Yeast cells co-expressing SnRK1α1-BD with STM, WUS, BLH8, BLH9 or ATH1-AD were grown on control medium lacking Leu and Trp (-L-W) or under increasingly selective media that were also lacking His (-L-W-H) to evaluate the strength of potential interactions. I observed that, in yeast cells, SnRK1α1 interacts with STM, WUS, BLH9 and ATH1 in the most selective medium tested, but it does not with BLH8 nor with the negative controls.

To further validate the Y2H data and determine if these SnRK1 interactions can also occur *in planta*, I next performed reciprocal coimmunoprecipitation (co-IP) experiments using Col-0 mesophyll cell protoplasts expressing SnRK1α1-HA with STM, WUS, BLH9 or ATH1 fused to GFP or with GFP alone as a negative control. SnRK1β2-GFP was used as a positive control for the interaction with SnRK1α1. Immunoprecipitation with an anti-GFP antibody and subsequent Western blot analyses revealed an interaction between SnRK1α1 and STM, WUS, BLH9, ATH1 and the

SnRK1β2 subunit (Fig. 1B), but not with the negative control, indicating that the interactions revealed by yeast-two-hybrid may also occur *in planta*. Taken together, our results suggest that SnRK1 interacts with key transcriptional regulators of SAM function, such as WUS, STM and some members of the BELL family.

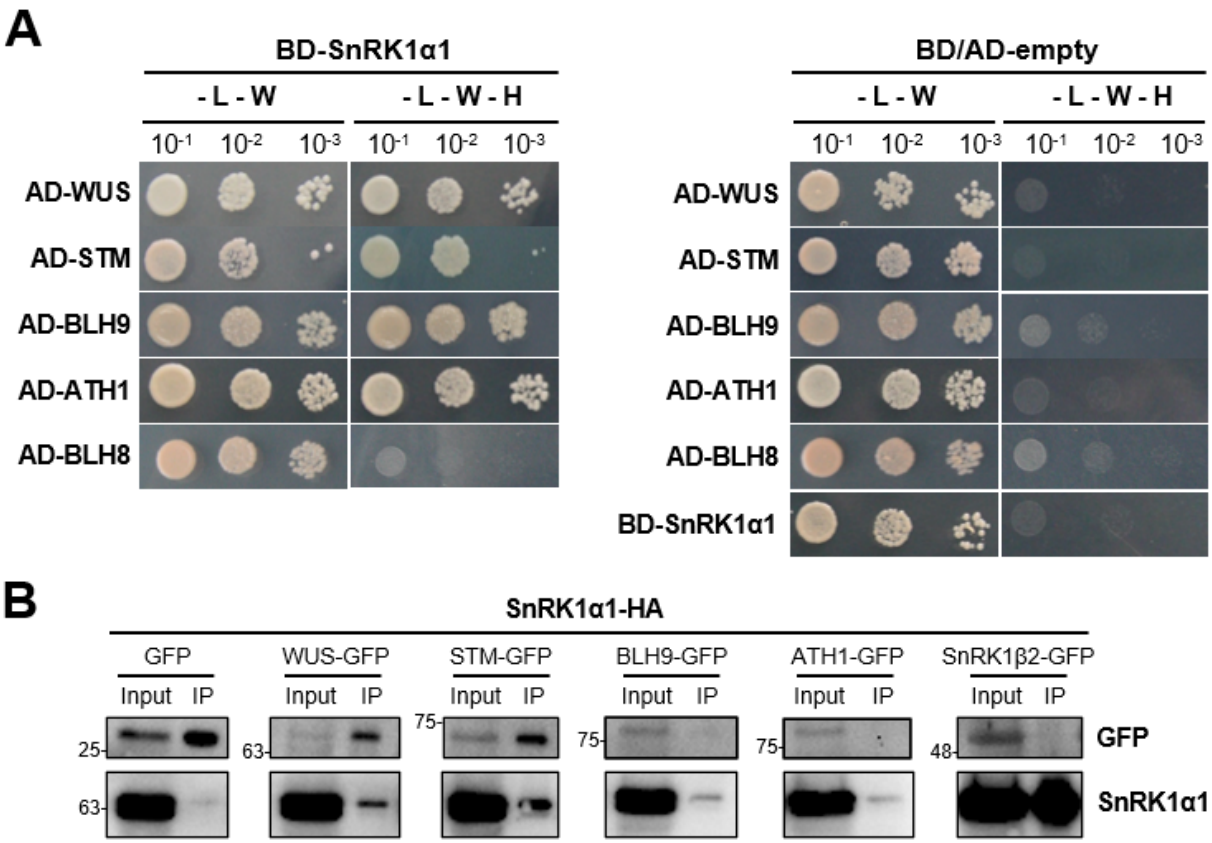


Figure 1. Interaction of SnRK1α1 with key regulators of SAM activity.

A, Yeast-two hybrid assays examining the interaction of SnRK1α1 with WUS, STM, BLH9, ATH1, and BLH8. Protein interaction was determined by monitoring yeast growth in medium lacking Leu, Trp and His (-L-W-H)

compared with control medium only lacking Leu and Trp (-L-W). *Left,* yeast growth in cells co-expressing WUS, STM, BLH9 or ATH1 with SnRK1α1. *Right,* negative controls of yeast transfected with the indicated AD/BD-constructs and the complementary BD/AD-empty vectors. *BD* and *AD*, DNA binding and activation domains of the GAL4 transcription factor, respectively. Increasing dilutions of transformed yeast cells are shown (10^{-1}, 10^{-2}, 10^{-3}). **B,** Co-immunoprecipitation (co-IP) experiments using Arabidopsis Col-0 mesophyll cell protoplasts co-expressing SnRK1α1-HA with STM, WUS, BLH9 or ATH1 fused to GFP or with GFP alone. GFP-tagged proteins were immunoprecipitated and co-immunoprecipitation of SnRK1α1 was assessed by immunoblotting with SnRK1α1-specific antibodies. SnRK1β2-GFP served as a positive control.

4.3.2. SnRK1 plays a role in STM/BELL activity

Local silencing of SnRK1α in the SAM leads to plant phenotypes highly reminiscent of those reported for STM and BLH8/BLH9/ATH1 loss of function (Chapter III; (Clark et al., 1996; Endrizzi et al., 1996; Landrein et al., 2015; Smith et al., 2004; Smith & Hake, 2003; Song et al., 2020). I therefore focused next on these TFs to explore the functional implications of the interaction of SnRK1 with these SAM regulators. To this end, I developed two reporters of STM activity by fusing the promoters of the previously described STM target genes *CUC1* and *IPT7* (Jasinski et al., 2005; Spinelli et al., 2011; Yanai et al., 2005) to *LUCIFERASE* (*LUC*) and used these reporters in transient protoplast-based assays (Yoo et al., 2007). As shown in Fig. 2A, STM expression triggered a 13-fold induction of the *CUC1* reporter that was not observed when a control plasmid was transformed. Expression of BLH9 alone,

on the other hand, had no impact on *CUC1::LUC* expression but potentiated the effect of STM on the reporter, supporting the cooperative interaction between these two TFs. Strikingly, the induction of *CUC1::LUC* in response to STM alone or to the combination of STM and BLH9 was fully suppressed by SnRK1α1. This repressive effect was no longer visible when a catalytically inactive SnRK1α1 variant (SnRK1α1_K48M; Baena-Gonzalez et al., 2007) was used, suggesting that SnRK1 may modulate the activity of these TFs *via* phosphorylation.

Co-expression of STM with other members of the BELL family, such as BLH8 (Fig. 2B) and ATH1 (Fig. 2C), led to a similar pattern on LUC activity. However, due to the low number of replicates in these experiments, the differences observed were not always statistically significant. Nevertheless, while SnRK1α1 seemed to counteract the impact of STM and BLH8 on *CUC1*::*LUC*, as previously observed with STM and BLH9, the combination of STM and ATH1 was fully recalcitrant to SnRK1α1 repression, suggesting that SnRK1 may affect STM activity differently depending on the nature of its BELL cofactor.

In contrast to *CUC1::LUC*, which was strongly induced by STM alone (Fig. 2A-C), the induction of *IPT7::LUC* required the presence of both STM and BLH9, highlighting the importance of the BELLs for STM function and specificity (Fig. 2D). However, similarly to *CUC1*, the induction of the *IPT7* reporter by STM and BLH9 was fully repressed by the overexpression of SnRK1α1.

Given the link between SnRK1 and the phosphorylation of proteins involved in translation (Belda-Palazón et al., 2020; Bruns et al., 2019; Cho et al., 2019; Nukarinen et al., 2016), we wondered whether the effect of SnRK1 in STM protein accumulation (Chapter III) was unspecific and could be explained by a general inhibition of translation that compromised STM-BELL

accumulation. To assess this, I analysed STM and BLH9 protein levels in the samples used for the reporter assays. As shown in Fig. 2E, Western blot analyses revealed only slightly lower levels of STM-BELL proteins in the samples co-expressing SnRK1, especially when both TFs were present.

This indicates that the repression observed in the reporter assays may not be triggered by a general downregulation of translation in the protoplast cells and may rather be due to SnRK1-triggered changes in STM-BELL activity.

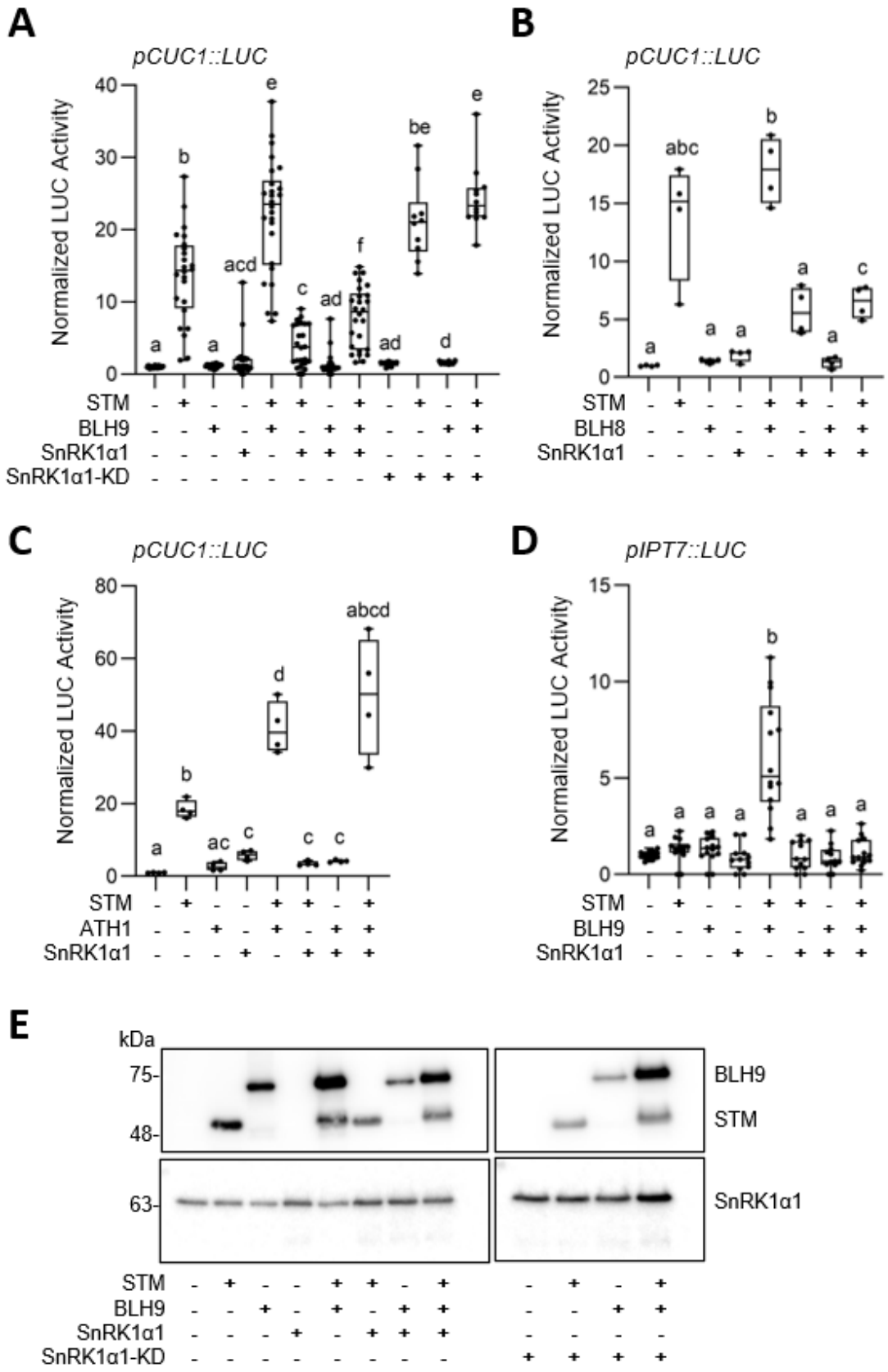

A
pCUC1::LUC
Normalized LUC Activity
STM
BLH9
SnRK1α1
SnRK1α1-KD
B
pCUC1::LUC
Normalized LUC Activity
STM
BLH8
SnRK1α1
C
pCUC1::LUC
Normalized LUC Activity
STM
ATH1
SnRK1α1
D
pIPT7::LUC
Normalized LUC Activity
STM
BLH9
SnRK1α1
E
kDa
75-
48-
63-
BLH9
STM
SnRK1α1
STM
BLH9
SnRK1α1
SnRK1α1-KD

Figure 2. **Impact of SnRK1 on STM/BELL activity.** Transcriptional activity, measured as the induction of the *pCUC1::LUC* (**A-C**) or *pIPT7::LUC* (**D**) reporters in Arabidopsis mesophyll protoplasts expressing STM, BLH8, BLH9, ATH1, and SnRK1α1 in the indicated combinations. SnRK1α1-KD, kinase dead SnRK1α1^{K48M} variant. Plots show normalized luciferase (LUC) activity values obtained from biologically independent protoplast preparations (A, *n*=26; B, *n*=14; C-D, *n*=4). Different letters denote statistically significant differences (Brown-Forsythe and Welch ANOVA, $p<0.05$). **E,** Immunoblot analyses of the samples described in (A) were used to evaluate STM and BLH9 protein accumulation in the absence or presence of SnRK1α1. STM and BLH9 were detected using an HA antibody whilst SnRK1α1 expression was confirmed using a SnRK1α1-specific antibody.

4.3.3. SnRK1 may potentially phosphorylate STM-BLH9

To evaluate whether SnRK1 affects STM-BLH9 activity through phosphorylation, I transiently coexpressed different combinations of SnRK1α1, STM and BLH9 in mesophyll protoplast cells, and ran these samples in a Phos-Tag SDS-PAGE that selectively retards phosphorylated proteins (Fig. 3) (Kinoshita et al., 2009). When expressed individually or together, the TFs showed a single band in the Phos-tag immunoblot, suggesting that they were present as single protein variants. However, when STM and BLH9 were co-expressed with SnRK1α1, a strongly retarded band became visible in the Phos-tag immunoblot, suggesting that one of the TFs was phosphorylated in a SnRK1α1-dependent manner. Importantly, this band was not detected when STM and BLH9 were co-expressed with the kinase

dead variant of SnRK1α1, lending further support for a potential phosphorylation of the STM- BLH9 complex to control STM activity.

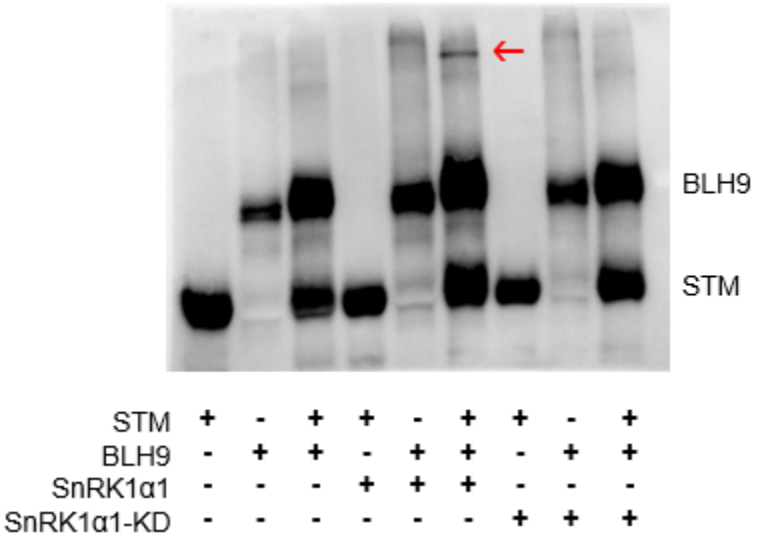


Figure 3. Analyses of STM/BELL phosphorylation by SnRKα1 *in vivo*. Total protein extracts of Arabidopsis mesophyll protoplasts expressing STM, BLH9, and SnRK1α1 in the indicated combinations were ran in Phos-tag gels to separate phospho-proteoforms from the non-phosphorylated protein. STM and BLH9 were thereafter detected by immunoblotting with an anti-HA antibody. SnRK1α1-KD, kinase dead SnRK1α1^{K48M} variant. Red arrow shows a slowly migrating protein band, potentially corresponding to a highly phosphorylated protein.

4.3.4. SnRK1 does not affect STM/BELL subcellular localization

Heterodimerization between STM and the BELL TFs has been shown to be essential for STM nuclear localization and thus, function (Aguilar-Martínez et al., 2015; Bellaoui et al., 2001; Bhatt et al., 2004; Cole et al., 2006; Liu et al., 2018; Rutjens et al., 2009). To examine the potential regulatory role of SnRK1 on STM-BELL activity and, given the importance of STM subcellular localization for its function, I investigated the potential impact of SnRK1α1 on the subcellular localization of these TFs. To this end, *Nicotiana benthamiana* (Nicotiana) leaves were infiltrated with *Agrobacterium tumefaciens* harbouring plasmids encoding for C-terminal fluorescent reporter fusions of GFP, RFP or mTurq and the infiltrated leaf segments were imaged 48h after infection (Fig. 4 and Supplementary Fig. 1). When expressed alone, SnRK1α1, BLH9, and ATH1 displayed a dual localization in the cytoplasm and the nucleus (Supplementary Fig. 1D-L). This contrasted with STM, which appeared to be mostly cytoplasmic (Supplementary Fig. 1A-C). However, when co-expressed with BLH9 or ATH1, STM became strongly enriched in the nucleus (Fig. 4A-D and J-M). This is consistent with previous reports (Cole et al., 2006; Rutjens et al., 2009) and supports a role for BELL TFs in the localization of STM to the nucleus. In addition, and as also observed in protoplasts, STM and BELL TFs accumulated to higher levels when expressed together, suggesting that the interaction between these TFs strongly increases their stability. SnRK1α1, on the other hand, did not cause obvious changes in the localization of the TFs, when expressed alone or in combination (Fig. 4 and Supplementary Fig. 1), suggesting that the repressive effect of SnRK1 on STM-BELL activity is unrelated to changes in their subcellular localization. However, the fact that co-expression with SnRK1α1 in this system led to a marked decrease in STM/BELL accumulation, precluded a precise analysis of TF localization. This contrasts with the observations in protoplasts (overnight incubation) and could

potentially be due to a general inhibition of translation upon prolonged expression of SnRK1α1 (2d).

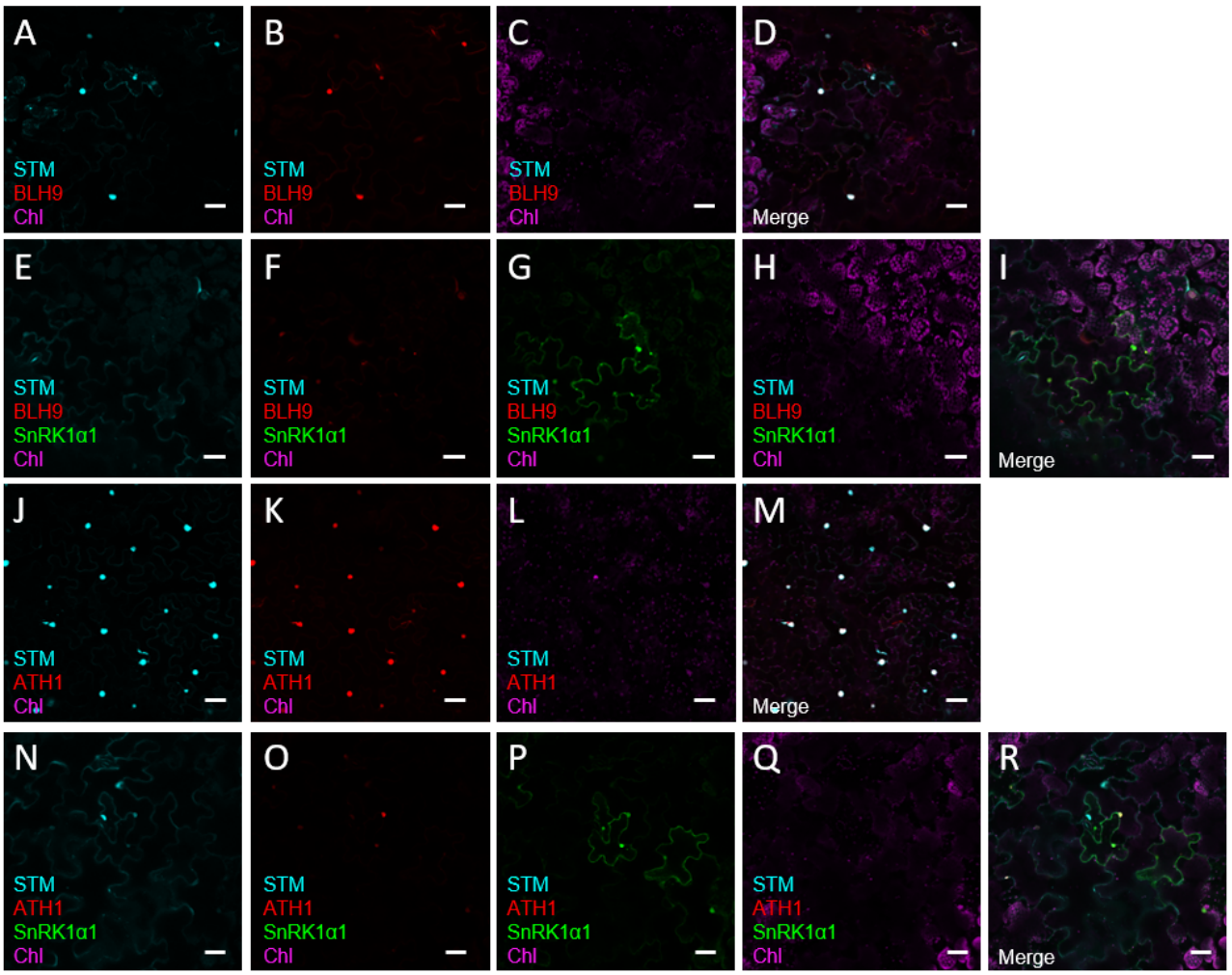


Figure 4. Impact of SnRK1 on STM-BELL subcellular localization. Transient expression of STM-mTurq (STM) with BLH9-RFP (BLH9, **A-D**) or ATH1-RFP (ATH1, **J-M**) in *Nicotiana benthamiana* leaves in the presence or absence of SnRK1α1-GFP (SnRK1α1; STM+BLH9, **E-I**; STM+ATH1-RFP).

Chlorophyll (Chl) autofluorescence signal was obtained for all the different constructs. Scale bars: 50 μm.

4.4. Discussion

Here we show that key regulators of SAM function, namely STM, WUS, BLH9 and ATH1, interact with the SnRK1α1 catalytic subunit in Y2H, and when co-expressed in Arabidopsis mesophyll cell protoplasts (Fig. 1). We further show that co-expression of SnRK1α1 with STM affects its activity and that whether SnRK1 is able to repress STM or not depends on the BELL partner that is available. Finally, the impact of SnRK1 on STM activity potentially involves phosphorylation of STM and/or its partners but does not appear to involve changes in the protein accumulation of these TFs nor in their subcellular localization.

The fact that SnRK1α1 impaired STM-BELL activity (Fig. 2A-D) without significantly altering their accumulation (Fig. 2E) was unexpected, given our previous observations that STM protein levels are markedly reduced during a 2-day dark treatment and are lower under optimal conditions in plants overexpressing SnRK1α1 (Chapter III). They were also surprising considering the strong negative impact of SnRK1α1 on STM/BELL accumulation in Nicotiana (Fig. 4 and Supplementary Fig. 1), which was in agreement with our previous observations *in planta* (Chapter III). One possible explanation for this apparent conflict could be the timescale of SnRK1 activation. Whilst reporter assays are typically performed overnight (12-16h), protein localization assays involve a minimum of 48h of protein expression. Similarly, our previous work compared steady-state STM protein levels in WT plants and in a mutant with constitutive SnRK1α1 overexpression as well as in plants treated with darkness for 48h (Chapter III). It is hence possible that, in the short-term, SnRK1α1 activation, leads to changes in the activity of STM through mechanisms unrelated to protein accumulation (see below). On the other

hand, sustained SnRK1α1 activity may trigger additional mechanisms to regulate STM-BELL function that involve changes in protein abundance. Nevertheless, the fact that SnRK1α1 clearly decreases the ability of STM-BELL to induce target gene expression without altering STM accumulation suggests that SnRK1 may regulate STM function in several ways, by affecting its protein levels (Chapter III) and by affecting its activity.

KNOX and BELL proteins can heterodimerize both *in vitro* and *in vivo* (Bellaoui et al., 2001; Bhatt et al., 2004; Byrne et al., 2003; Chen et al., 2003, 2004; Cole et al., 2006; Hackbusch et al., 2005; Muller et al., 2001; Smith & Hake, 2003). These interactions are essential for their function, as they promote the nuclear localization of both TFs (Bhatt et al., 2004; Cole et al., 2006; Kimura et al., 2008; Rutjens et al., 2009) and increase their DNA binding affinity (Kim et al., 2013; Smith et al., 2002). Hence, it is plausible that SnRK1 could modulate these interactions and thereby STM-BELL function. Consistent with this hypothesis, expression of STM alone or with specific BELLs induced the*CUC1::LUC* reporter of STM activity and this could be suppressed by the co-expression of SnRK1α1 (see below for further details and discussion). On the other hand, although we could confirm that STM is not localized to the nucleus by default, and that its nuclear accumulation requires co-expression of the BELL factors (Cole et al., 2006), SnRK1α1 did not cause any obvious change in STM nor BELL localization in Nicotiana. These results may suggest that SnRK1 repression of STM-BELL activity is unrelated to the subcellular localization of the TFs. Nevertheless, SnRK1 could still play a role in STM-BELL regulation by affecting the DNA binding specificity of these dimers. Such a mode of regulation by SnRK1 has been described for the C- and S1- subclass of bZIP TFs, whose heterodimerization is important for their function as downstream effectors of SnRK1 signalling (Baena-González et al., 2007; Mair et al., 2015). Indeed, phosphorylation of

bZIP63 (C-class) by SnRK1 changes its dimerization preferences, favouring its interaction with a specific member of the S1 class over another and thereby influencing the capacity of bZIP63 to recognize and activate specific promoters (Mair et al., 2015).

The fact that SnRK1 acts as a strong repressor of STM function, which could not be reverted by the presence of BLH8 or of BHL9, supports a direct role of SnRK1 in STM regulation. This may indicate that SnRK1 phosphorylates STM, thereby changing some aspect (such as its binding affinity, localization, or protein stability) that ultimately impacts on its function. However, it is also possible that the effect of SnRK1 on STM activity is indirect and that it relies on the BELL TFs or on other factors that influence STM function and that are naturally expressed in protoplasts. Such potential factors could for example be BLH4 or BLH6, both of which are highly expressed in rosette leaves (https://bar.utoronto.ca/eplant/; Toufighi et al., 2005). Thus, SnRK1 could phosphorylate BELL TFs as a means to regulate STM function through changes in the formation of specific STM-BELL complexes and/or in their capacity to bind DNA. This is consistent with our results that STM alone is capable of inducing *CUC1::LUC* but not *IPT7::LUC* expression (Fig. 2A-D). This is further supported by the fact that, contrary to BLH8 and BLH9, ATH1 is able to sustain STM function in protoplasts in the presence of SnRK1 (Fig. 2C), which suggests that SnRK1 may modulate STM function through regulation of specific BELLs (BLH8 and BLH9) but not of others (ATH1). This is also in agreement with reports showing that, despite performing similar functions to those of BLH8 and BLH9 in the establishment and maintenance of the SAM (Rutjens et al., 2009), ATH1 has also different and sometime opposite roles to other BELLs, for example in the regulation of flowering time (Kanrar et al., 2008; Proveniers et al., 2007; Smith et al., 2004) and inflorescence length (Cole et al., 2006).

Therefore, the mechanisms linking SnRK1 to STM regulation remain unclear. On one hand, the repressive effect of SnRK1α1 on STM alone, points at STM being a direct SnRK1 target. On the other hand, the fact that, contrary to BLH8 and BLH9, ATH1 could sustain STM function even when co-expressed with SnRK1α1 prompts the BELLs as the actual targets of this kinase. However, it could still be argued that the direct interaction between STM and SnRK1α1, also observed in Y2H, could be blocked by the binding of ATH1 to STM, potentially by masking an important domain of STM that is recognized by SnRK1. Likewise, ATH1 could act as a dominant negative BELL, preventing the interaction between STM and endogenously expressed BELLs that are the actual SnRK1 targets. A scenario where SnRK1 regulates STM function through the interaction with specific BELLs is further supported by the Phos-tag immunoblot analyses (Fig. 3) which revealed a slowly migrating band (potentially corresponding to a highly phosphorylated protein) when STM and BLH9 were co-expressed with SnRK1α1, and which was also weakly visible when expressing BLH9 alone. However, whether this potential phosphorylation event(s) corresponds to STM or to BLH9 is unclear. Future analyses should identify which TF(s) and residue(s) are modified and test if this band is still present in the presence of other BELLs, such as ATH1.

To conclude, our results suggest a role for SnRK1 in the SAM through direct interaction with key meristem regulators, such as STM and the BELL TFs and potentially with other key keepers of the SAM, such as WUS. SnRK1 represses the activity of STM, possibly through phosphorylation of STM or its BELL TF partners. This may influence the formation of heterodimeric complexes between STM and specific BELLs and thus, impact on STM activity. However, further experiments will be needed to identify the direct targets of SnRK1 and how those fit in the complex regulation of STM and thus, the SAM.

4.5. Material and Methods

Protoplast assays

Protoplast transient expression assays were carried out as previously described, using freshly isolated mesophyll cells from mature fully expanded Arabidopsis leaves (Baena-González et al., 2007; Yoo et al., 2007). Following transfection, protoplasts were incubated for 16h under light conditions (25-30 µmol m^{-2} s^{-1} and 23°C).

For constructs for the overexpression of SnRK1α1, STM, BLH9, ATH1 and BLH8, the corresponding coding sequences were amplified from seedling cDNA with primers introducing restriction sites for cloning into pHBT95 (with or without a C-terminal HA tag) or Gateway recombination sites for cloning into p2GWC7 vector (Karimi et al., 2005)(with a C-Terminal GFP tag; see primer list below). STM signaling was monitored using a *pCUC1:LUC* or a *pIPT7:LUC* reporter (Jasinski et al., 2005; Spinelli et al., 2011; Yanai et al., 2005) and a *pUBQ10::GUS* reporter as transfection control. A control DNA yielding no protein expression was used as a negative control and to adjust the total amount of DNA per transfection to 20 µg. All effector constructs (SnRK1α1 and the TFs) were added at a 1:1 ratio. For all samples, the LUC signal was normalized by the GUS signal to account for potential differences in transfection efficiency (Baena-González et al., 2007; Yoo et al., 2007).

For coimmunoprecipitation (Co-IP) assays, the indicated combinations of constructs (at a 1:1 ratio) were used to transfect 1 ml of mesophyll cell protoplasts. Frozen cell pellets were lysed in 500 µl of lysis buffer [150 mM NaCl, 1% (v/v) Triton-X, 50 mM Tris-HCl (pH 8.0), 3 mM DTT, supplemented with 50 µM MG132, 1:20 Complete EDTA-free Protease Inhibitor Cocktail (Roche), and 1:500 Phosphatase Inhibitor Cocktails 2 and 3 (Sigma-Aldrich)]. The cleared lysate was incubated with 30 µl of super-paramagnetic µMAC

beads coupled to a monoclonal anti-GFP antibody (Miltenyi Biotec) for 2h at 4°C with gentle rocking. Purified immunocomplexes were eluted in Laemmli buffer, boiled, run in an 10% SDS-PAGE gel, transferred to PVDF membranes (wet transfer at 4°C, 100 V, 70 min) and analysed by immunoblotting using antibodies against GFP (1/1000, Roche, #11814460001), HA (1/1000, Roche, #11867423001), and SnRK1α1 (Belda-Palazón et al., 2020; used at a 1:2000 dilution).

For immunoblot analyses of samples used in the reporter assays, the protoplast pellets of replicate reporter assay samples expressing the indicated protein combinations were boiled in Laemmli buffer. Proteins were separated by SDS-PAGE in 10% acrylamide gels, transferred to PVDF membranes (wet transfer at 4°C, 100 V, 70 min) and probed using 1:1000 or 1:2000 dilutions of HA or SnRK1α1 antibodies, respectively.

To access STM and BLH9 phosphorylation, samples used in the reporter assays were prepared as previously described for the immunoblotting and were run in a 8% acrylamide phos-tag SDS-PAGE gel (25 mM Phos-Tag ligand [Wako] and 25 mM MnCl2) (Kinoshita et al., 2009) and immunoblot with an anti-HA antibody. The Phos-Tag ligand selectively retards phosphorylated proteins.

For immunodetection, secondary antibodies conjugated with horseradish peroxidase (AffiniPure goat anti-rabbit (#111035144) or anti-rat IgG (H+L) (#112035167); Jackson ImmunoResearch; www.jacksoniummuno.com) were used at 1:10000 dilutions. Chemiluminescence-based detection of peroxidase activity was performed using a SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

Yeast-two-hybrid

For Y2H assays, the coding sequence of SnRK1α1 was cloned into the pGBKT7 vector (Rodrigues et al., 2013) and the coding sequences of STM, BLH9, ATH1 and BLH8 were cloned into the pGADT7 vector using the primers listed below. Y2H assays were performed as described (Saez et al., 2008). The pGBKT7- SnRK1α1 constructs harboring the GAL4 DNA-binding domain (BD) were faced with the pGADT7-TF constructs harbouring the GAL4 activation domain (AD). As controls, pGBKT7-SnRK1α1 subunit and pGADT7-TF constructs were co-transformed with the respective complementary empty vectors.

Infiltration of *Nicotiana benthamiana* leaves

For protein expression in *Nicotiana benthamiana* leaves, the coding sequences of SnRK1α1, STM, BLH9, ATH1 and BLH8 were cloned into pK7m34GW vectors harboring C-terminal RFP, mTurq, or GFP tags using the primers listed below. *Agrobacterium tumefaciens*, strain GV3101 (pMP90) was transformed with the indicated combinations of constructs (at a 1:1 ratio). Clones were screened by colony PCR and positive clones were selected for infiltration of Nicotiana leaves. Briefly, 1 mL of fully grown bacterial culture was pelleted by centrifugation at room temperature. The pellet was washed once in infiltration buffer [10 mM $MgCl_2$ and 10 mM MES] and once in infiltration buffer supplemented with 100 µM acetosyringone and was finally resuspended in 1 mL of the same acetosyringone- supplemented buffer. The resulting bacterial suspension was diluted to a final OD600 of 0.5. This Agrobacterium solution was used to infiltrate tobacco leaves through the stomata of the lower epidermis, by using a 1 mL syringe without needle. For co-infiltration experiments, bacterial strains containing their respective constructs were mixed (at a 1:1 ratio) prior to leaf infiltration. Agrobacteria

containing the p19 viral suppressor were employed in all infiltration experiments, at an OD600 of 0.5.

Imaging

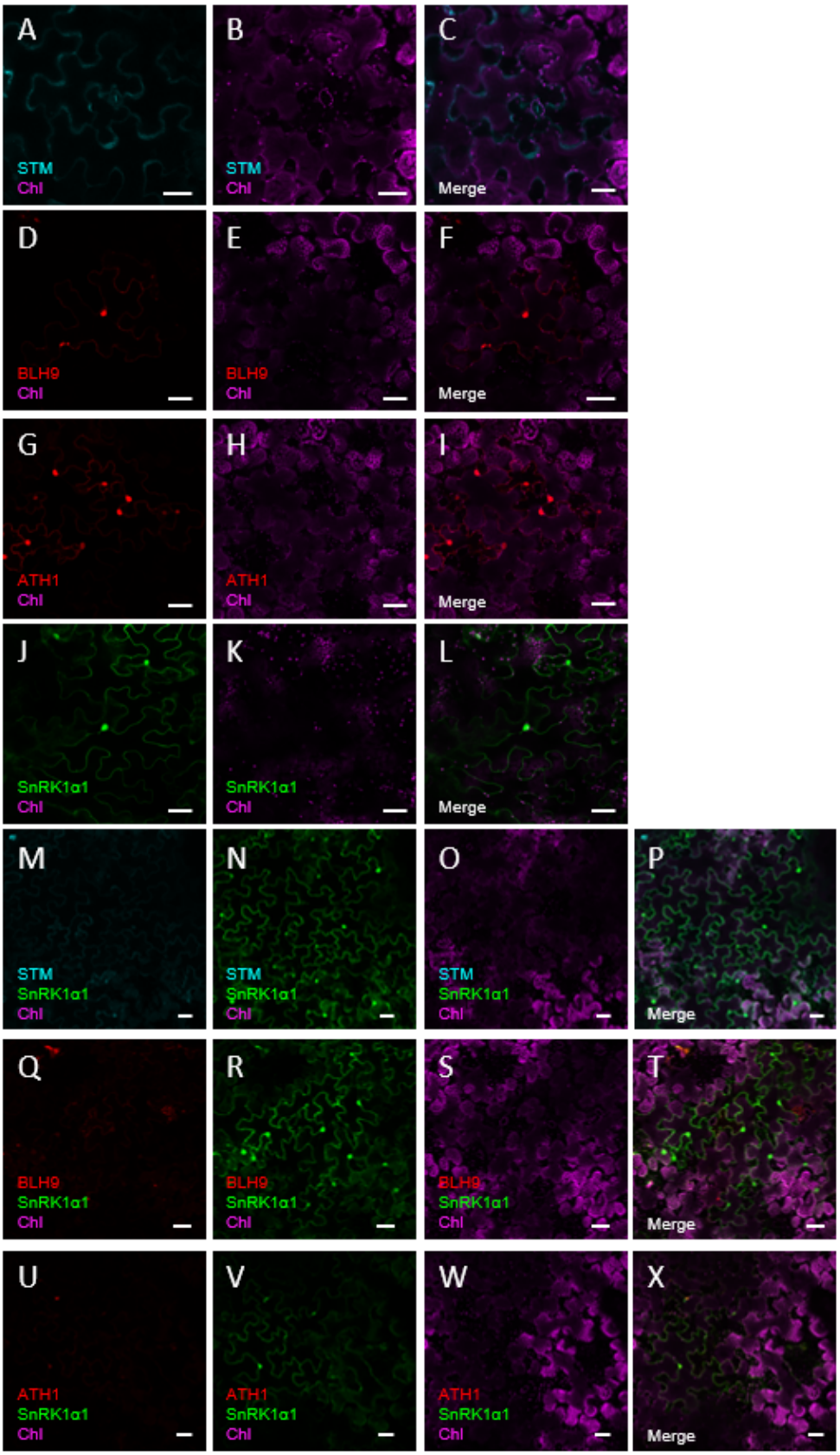
Fluorescence was analysed 48h after tobacco leaf infiltration. Leaf circles were randomly sampled from the infected leaves and mounted in water for microscopic observation. Imaging was performed on a Zeiss LSM 980 under a 10x or 20x objective. The laser lines 405 nm, 488 nm, 561 nm, and 639 nm were used and their spectral detection adjusted for the emission of mTurq, GFP, RFP, and far-red (chlorophyl), respectively.

List of primers used in this study

Nicotiana benthamiana transient assays	
STM-AttB2R-F	GGGGACAGCTTTCTTGTACAAAGTGGCT ATGGAGAGTGGTTCCAACAG
STM-AttB3-R	GGGGACAACTTTGTATAATAAAGTTGC AAGCATGGTGGAGGAGATGTG
WUS-AttB2R-F	GGGGACAGCTTTCTTGTACAAAGTGGCT ATGGAGCCGCCACAGCATCAGC
WUS-AttB3-R	GGGGACAACTTTGTATAATAAAGTTGC GTTCAGACGTAGCTCAAGAG
BLH9-AttB2R-F	GGGGACAGCTTTCTTGTACAAAGTGGCT ATGGCTGATGCATACGAGC
BLH9-AttB3-R	GGGGACAACTTTGTATAATAAAGTTGC ACCTACAAAATCATGTAG
ATH1-AttB2R-F	GGGGACAGCTTTCTTGTACAAAGTGGCT ATGGACAACAACAACAACAAC
ATH1-AttB3-R	GGGGACAACTTTGTATAATAAAGTTGC TTTATGCATTGCTTGGCTC
KIN10-AttB2R-F	GGGGACAGCTTTCTTGTACAAAGTGGCT ATGTTCAAACGAGTAGATG
KIN10-AttB3-R	GGGGACAACTTTGTATAATAAAGTTGC GAGGACTCGGAGCTGAGC

Y2H	
WUS _attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGCCGCCACAGCATCAGC
WUS _attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTGTTCAGACGTAGCTCAAGAG
STM _attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGAGAGTGGTTCCAACAG
STM _attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTAAGCATGGTGGAGGAGATGTG
ATH1 _attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGACAACAACAACAACAAC
ATH1 _attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTTTTATGCATTGCTTGGCTC
BHL8 _attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGATATGATAAAACCAG
BHL8 _attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTACCCACAAAGTCATGAAAC
BHL9 _attB1	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTGATGCATACGAGC
BHL9 _attB2	GGGGACCACTTTGTACAAGAAAGCTGGGTTACCTACAAAATCATGTAG

4.6. Supplementary Figures


A
STM
Chl
B
STM
Chl
C
Merge
D
BLH9
Chl
E
BLH9
Chl
F
Merge
G
ATH1
Chl
H
ATH1
Chl
I
Merge
J
SnRK1α1
Chl
K
SnRK1α1
Chl
L
Merge
M
STM
SnRK1α1
Chl
N
STM
SnRK1α1
Chl
O
STM
SnRK1α1
Chl
P
Merge
Q
BLH9
SnRK1α1
Chl
R
BLH9
SnRK1α1
Chl
S
BLH9
SnRK1α1
Chl
T
Merge
U
ATH1
SnRK1α1
Chl
V
ATH1
SnRK1α1
Chl
W
ATH1
SnRK1α1
Chl
X
Merge

Supplementary Figure 1. Impact of SnRK1 on STM-BELL subcellular localization. Transient expression of STM-mTurq (STM; **A-C**), BLH9-RFP (BLH9, **D-F**), ATH1-RFP (ATH1, **G-I**) in the absence or presence of SnRK1α1-GFP (STM; **M-P**; BLH9, **Q-T**; ATH1, **U-X**) in *Nicotiana benthamiana* leaves. **J-L**, Expression of SnRK1α1-GFP alone. Chlorophyll (Chl) autofluorescence signal was obtained for all the different constructs. Scale bars: 50 μm.

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CHAPTER V

General Discussion

5. General Discussion

For all organisms, adjusting growth to the available resources is essential for cell maintenance, growth and division, and ultimately survival. This is of particular importance for sessile organisms like plants, which rely on the modification of physiology and growth for coping with the soil and weather conditions they encounter. The availability of sugars and other nutrients under favourable conditions is sensed through dedicated and highly conserved signalling pathways that accelerate growth and developmental progression, determining when critical events such as germination or flowering occur. Conversely, under unfavourable conditions, sugar production through photosynthesis is compromised, leading to growth restrictions, and the activation of nutrient recycling processes to help cells survive.

Growth and developmental decisions take place in the meristems where stem cell proliferation is finely balanced with cell differentiation in accordance with the environment. However, how environmental information is translated into changes in meristem activity is poorly understood.

This thesis investigated this question, using as a model the shoot apical meristem (SAM) of *Arabidopsis thaliana*. The work presented here demonstrated that environmental cues, such as light, promote the accumulation of the transcriptional regulator SHOOT MERISTEMLESS (STM), a key keeper of SAM activity. The effect of light appeared to be indirect *via* photosynthesis-mediated sugar production and was counteracted by the Sucrose Non-Fermenting 1 (SNF1) - related kinase1 (SnRK1) (Chapter III). On the other hand, local silencing of SnRK1 in the SAM led to a marked reduction in STM levels and to phenotypes highly reminiscent of those described for partial STM loss-of-function mutants, showing that SnRK1 is also essential for keeping SAM integrity (Clark et al., 1996; Endrizzi, Karin K.

et al., 1996; Landrein et al., 2015; Song et al., 2020). The mechanisms underlying the impact of SnRK1 on STM accumulation were further investigated, revealing a physical interaction of the SnRK1 catalytic subunit α1 with STM as well as with several other central SAM regulators, such as, BEL1-like 9 (BLH9), ARABIDOPSIS THALIANA HOMEOBOX 1 (ATH1), and WUSCHEL (WUS). The SnRK1 kinase was further found to strongly inhibit STM activity, potentially *via* phosphorylation of STM and/or its transcription factors (TF) partners.

5.1. Sugars promote the accumulation of STM, a gatekeeper of SAM activity

Perceiving environmental cues, such as light, and translating that information into adequate meristematic activities, is essential for plant adaptation to the environment and thus, proper plant development and physiology. Indeed, it has been recently shown that light, sugars, mineral nutrients, and oxygen can influence meristem function (Chapter II). For example, light modulates SAM activity through its effect on different phytohormones such as cytokinin (CK), impacting WUS accumulation and thereby shaping plant growth at several stages of development (Pfeiffer et al., 2016; Yoshida et al., 2011). Similar to the WUS protein, whose accumulation is induced by light (Pfeiffer et al., 2016), STM protein levels in the SAM are also directly correlated with light intensity, with STM levels increasing under higher irradiances (Chapter III). By contrast, STM accumulation is strongly repressed in the dark, and accordingly, STM activity is impaired.

Nevertheless, light was not sufficient to sustain STM levels when the meristems were excised, suggesting that a light-related signal is sensed

outside the meristem and systemically transmitted to the SAM and that this communication is disrupted upon excision of the inflorescence (Chapter III). Regulation of SAM activity by systemic signals has indeed been established for other nutrients like nitrogen. Nitrate can act as a signal itself, but long-distance nitrogen signalling is also mediated by CK (Zhang et al., 2020). Nitrate availability in the soil promotes the production of CK precursors by phosphate-isopentenyl transferases (IPTs) enzymes in the root. These CK precursors are then translocated to the shoot, where they are converted to active CK through the LOG enzymes, promoting CK signalling and thus, *WUS* expression in the SAM (Landrein et al., 2018). In the case of STM, CK was not capable of sustaining STM protein levels in excised meristems (Chapter III), suggesting that, unlike *WUS*, STM may not respond to CK-mediated nitrogen signals. Furthermore, preliminary experiments with plants subjected to different fertilization regimes revealed no obvious differences in STM-VENUS levels (results not shown). However, these experiments should be replicated in the future and should include e.g. *pWUS::GFP* plants as a positive control for the fertilization treatment. It is also plausible that STM expression is influenced by other signals that are external to the meristem. Future experiments should address this e.g. by supplementing excised STM-VENUS SAMs with different nutrient combinations.

In addition to the CK-mediated systemic regulation, WUS was shown to be regulated by light, which directly promoted and was sufficient to sustain WUS protein expression in young seedlings (Pfeiffer et al., 2016). This contrasts with STM, whose accumulation was also enhanced by light, but light was not sufficient to sustain STM levels *in vitro,* likely acting indirectly through photosynthesis-dependent sugar production (Chapter III; see below). This may indicate that WUS and STM are modulated by light through different pathways (light and sugar signalling, respectively), potentially to enable a

more robust maintenance of the stem cell niche through the integration of two complementary signals (light and sugars). It is also possible that the differences between WUS and STM regulation relate to the developmental stage of the plants used in the two studies (Pfeiffer et al., 2016; Chapter III). Light plays key regulatory roles during early seedling development (Fankhauser & Chory, 1997; Roeber et al., 2021; Teixeira, 2020) and, whilst being an absolute requirement for meristem activation and the induction of cell division in young vegetative meristems (Li et al., 2017; Pfeiffer et al., 2016), it may not play such a dominant role in adult inflorescence meristems (Chapter III). To investigate whether light alone can sustain WUS expression in inflorescence meristems or whether, similarly to STM, this requires sugar production through photosynthesis, WUS reporter lines could be analysed in the future using the experimental set-up described in this thesis. Alternatively, the WUS and STM differences could be caused by the different manipulation of the plants in the two studies. One could argue that the light and sugar-independent signal that sustains WUS expression (and potentially STM) in intact seedlings (Pfeiffer et al., 2016) is lost when the meristems are excised in the *in vitro* and semi–*in vitro* systems employed in this thesis. However, the fact that, at least for STM, sugar supplementation could rescue STM expression (Chapter III), suggests that this is not the case and that the effect of light is indirect through sugars, which become rapidly depleted in the excised inflorescence.

Supporting this, dark-treated plants suffered a rapid decline in sugar content in the meristem, which could potentially explain the decrease in STM levels also observed under these conditions. The correlation between the sugar content in the SAM and STM protein abundance together with the fact that sucrose supplementation was sufficient to sustain STM levels in excised meristems, indicates that sugars are sensed locally in the SAM. The regulation

of STM by sugars is in line with several recent studies linking nutrient signals to the expression of meristem factors and SAM activity and support the emerging view that meristem activity is strongly influenced by the availability of major nutrients. Accordingly, WUS expression in the SAM is tightly balanced by sugars, such as sucrose (Pfeiffer et al., 2016) and by nitrogen *via* CK signalling (Landrein et al., 2018), ultimately driving SAM maintenance and activity. The fact that both carbon and nitrogen signals contribute to SAM activity is also consistent with the fact that carbon and nitrogen metabolism are tightly coordinated (Lastdrager et al., 2014; Vidal et al., 2020; Wingler, 2017). Besides sucrose, glucose also induces *WUS* expression in young seedlings (Pfeiffer et al., 2016). Furthermore, this induction was shown to be partially dependent on the TOR complex, similarly to the glucose-driven activation of the root meristem. Interestingly, sucrose seems to promote *WUS* expression in the dark at higher levels than glucose (Pfeiffer et al., 2016), suggesting that different sugars could impact the expression of this TF expression at different levels. For STM, sucrose could sustain its protein levels in the dark in a dose-dependent manner, but whether this effect is specific to sucrose or is common to other metabolizable sugars remains to be assessed.

There is a strong positive correlation between sucrose and treahalose-6-phosphate (Tre6P) levels, that has been confirmed in different tissues, developmental stages (Carillo et al., 2013; Sulpice et al., 2014; Wahl et al., 2013; Wingler et al., 2012; Yadav et al., 2014) and species (Debast et al., 2011; Henry et al., 2014). Similarly to the glucose-insulin system of mammals, Tre6P levels rise in response to sucrose and regulate carbon and nitrogen metabolism to restore sucrose levels to an optimum. Sink tissues typically have high Tre6P:sucrose ratios and this is thought to promote high metabolic activity and active growth (Lunn et al., 2014). An example of this are the 8-fold higher ratios of the inflorescence meristems as compared to rosettes grown

under the same conditions (Lunn et al., 2014; Wahl et al., 2013; Yadav et al., 2014), These observations prompt Tre6P as a potential mediator of the sucrose signal and thereby as a prime candidate in the modulation of meristem factors and SAM activity. Indeed, the *in vitro* assays performed in this thesis could be used to test how different sugars, such as Tre6P or glucose are differently perceived in the SAM, impacting the levels of STM or other key regulators, and hence their activity.

5.2. Molecular mechanism

One important aspect unveiled in this thesis is that the effect of light, and thus sugars, on STM, is at the protein level, as high irradiance and sugar provision promoted STM protein accumulation without altering *STM* transcript levels (Chapter III). This suggests that photosynthesis-derived sugars control STM accumulation through mechanisms that directly influence STM protein levels, potentially by adjusting STM protein translation rates and/or stability and thus, STM function.

One possibility explored in more depth in this thesis, is that energy signals are sensed in the SAM by the SnRK1 protein kinase, which in response to energy depletion, mobilizes reserves and turns off energy-consuming processes like growth. Indeed, the ubiquitous overexpression of the SnRK1α1 catalytic subunit was found to reduce STM accumulation under high irradiance and this could not be explained by reduced sugar levels in the SAM of the overexpressor plants (Chapter III). Furthermore, SnRK1 was found to be enriched in the SAM, where it was activated when plants were subjected to unfavourable light conditions (Chapter III). This suggests that SnRK1 acts locally in the SAM to modulate meristem activity in accordance with the sugar

status. Such conclusion was further supported by the severe developmental phenotypes resulting from *SnRK1α* silencing locally in the SAM (see next section).

How does SnRK1 activity affect STM accumulation? The fact that STM, WUS, BLH9 and ATH1, interact with the SnRK1α1 catalytic subunit (Chapter IV) points that SnRK1 may modulate SAM maintenance and activity through direct interaction with key regulators of SAM activity. Supporting this idea, transient reporter assays revealed a strong inhibition of STM function by the SnRK1 kinase (Chapter IV). However, this was not accompanied by a clear reduction in STM accumulation, as expected from the lower STM levels of SnRK1α1 overexpressor plants (Chapter III). This suggests that SnRK1 regulates STM in several ways. On one hand, SnRK1 modulates STM protein accumulation through unknown mechanisms that likely affect its translation and/or stability. Such regulation may require other meristem factors and may not be a fast response, since co-expression of SnRK1 with STM in protoplasts did not cause any notable decrease in STM levels (Chapter IV), whilst such decrease was clear in SnRK1α1 overexpressor plants (Chapter III). On the other hand, SnRK1 appeared to affect STM activity independently of its accumulation. Furthermore, this involved phosphorylation, as the use of a kinase-dead SnRK1α1 variant in these assays abrogated the inhibitory effect observed with the non-mutated kinase. However, whether such phosphorylation occurs on STM or on its BELL partners, is thus far unknown. Future work could employ *in vitro* kinase assays to investigate if STM and/or BELL TFs are direct SnRK1 substrates and if so, to identify the actual target residues by mass spectrometry. Additional evidence for this phosphorylation occurring on STM and/or the BELLs could be obtained from the inclusion of phosphatase-treated samples as controls in the Phos-tag gels and by having different tags in the STM and the BELLs to enable the assignment of band

shifts to a specific TF. This would confirm that the mobility shift observed in STM/BELLs in the presence of active SnRK1α1 (Chapter IV) corresponds indeed to phosphorylation events on one of these proteins. The identity of the identified phosphoresidues could be confirmed and their functional relevance subsequently assessed by generating the corresponding phosphomutant variants (mutation of the S/T residue to non-phosphorylatable A). These variants could be then compared to the WT proteins in *in vitro* kinase assays and in the reporter assays.

What could be the mechanism(s) underlying the SnRK1-induced decrease in STM activity? The KNOX and BELL are known to form heterodimers, and the formation of these dimers is fundamental for their functions, since they promote their relocalization to the nucleus and modulate their DNA binding affinity (Bhatt et al., 2004; Cole et al., 2006; Kim et al., 2013; Kimura et al., 2008; Rutjens et al., 2009; Smith et al., 2002). Thus, hypothetically, SnRK1 could modulate these interactions and thereby STM-BELL function in the SAM, through the phosphorylation of STM or the BELL TFs. Previous work described the presence of a highly conserved S residue (S272) in the ELK domain of STM whose phosphorylation modulates the interaction with its partner proteins and hence STM nuclear localization (Aguilar-Martínez et al., 2015). Towards the end of this work, I generated an STM variant that cannot be phosphorylated on this residue (S272A) to evaluate in reporter assays whether this phosphorylation impacts STM activity and its repression by SnRK1. Preliminary results, nevertheless, revealed no effect of this mutation on STM activity in these assays (results not shown). This may indicate that the mechanism by which SnRK1 impacts STM activity does not involve changes in its subcellular localization but rather a process downstream of it, like DNA binding. This would be in line with the results of protein localization in Nicotiana leaves, where SnRK1 did not cause obvious

changes in the localization of the TFs (Chapter IV). The lack of effect of the S272A mutation in the reporter assays may also point that the repressive effect of SnRK1 targets other regions of STM, or even the BELLs.

Indeed, preliminary *in silico* analyses of STM and BELL protein sequences with a SnRK1 consensus motif prediction tool (Carianopol et al., 2020) reveal potential phosphorylation target sites in both TFs (not shown). Therefore, future studies could explore the functional relevance of these potential phosphorylation events by testing the corresponding phosphovariants in the protoplast reporter assays. Nevertheless, the relatively large amount of such predicted sites makes this approach still not ideal, as it could require the generation of a vast number of combinatorial mutations. Therefore, the use of *in vitro* kinase assays followed by MS/MS analyses would still remain the favoured approach to disentangle the actual SnRK1 target residues.

By targeting the BELLs, SnRK1 could modulate specific STM functions associated with its binding partners and not others. This would be compatible with the results from the protoplast assays, where the repression of STM by SnRK1α1 was seen only in the presence of BLH8/BLH9 but not in the presence of ATH1 (Chapter IV). Phosphorylation of the BELL could alter the properties of the STM-BELL heterodimer in a way that the recognition of target promoters and/or DNA binding is altered.

5.3. SnRK1 plays a dual function in the SAM

The results discussed in the previous sections indicated that SnRK1 is a negative regulator of STM and suggested that this regulation may occur locally in the SAM. To discriminate between local and systemic effects in a more

direct manner, the SnRK1α subunits were silenced in the meristem by expressing specific amiRNAs under the *STM* promoter. This caused a marked decrease in STM accumulation and a variety of developmental abnormalities similar to the ones described for partial STM loss-of-function (Clark et al., 1996; Endrizzi et al., 1996; Landrein et al., 2015; Song et al., 2020). These results demonstrate that SnRK1 plays a critical role in the SAM, being required for proper SAM organization and function.

Nevertheless, the outcome of SnRK1 silencing on STM protein accumulation was highly unexpected, as it was similar to what was observed when SnRK1 is ubiquitously overexpressed. However, there are important differences between these two systems. First, SnRK1α1 overexpressors do not show overt SAM defects or developmental anomalies similar to those observed in the amiRNA lines. This is line with what was previously observed in plants overexpressing SnRK1α1, which displayed mostly developmental delays rather than defective organ development and arrangement (Baena-González & Hanson, 2017; Jamsheer et al., 2021). Second, *STM* transcript was not altered in the SnRK1α1 overexpressors, whilst it was strongly reduced in the most severe amiRNA lines. Altogether these results indicate that SnRK1 plays a dual role in the SAM, a more direct one on STM, that involves protein accumulation and activity, as previously discussed, and a more indirect one through unknown factors that ultimately leads to decreased STM transcript and protein accumulation.

Sugars are known repressors of SnRK1, but at the same time promote STM protein accumulation. This may seem in conflict with the phenotypes and the strong STM protein depletion found when down-regulating SnRK1 locally in the SAM. Nevertheless, although SnRK1 is generally considered a growth repressor that is activated in response to energy depletion for example during stress, several lines of evidence suggest that this is an oversimplification and

that SnRK1 is also essential for growth (Baena-González & Lunn, 2020; Margalha et al., 2019). Transient silencing of *SnRK1 via* virus-induced gene silencing causes full growth arrest (Baena-González et al., 2007). Furthermore, double *snrk1α1 snrk1α2* mutants could thus far not be recovered, suggesting that complete loss of SnRK1α is embryo lethal (Ramon et al., 2019).

The essentiality of SnRK1 could for example be related to its involvement in the cell cycle. Indeed, it has been shown that in Arabidopsis, SnRK1 plays an important role in cell proliferation by inhibiting the function of a cyclin-dependent kinase inhibitor, KRP6 (Kip-related protein 6) through phosphorylation (Guérinier et al., 2013). Such an effect on cell cycle progression could indirectly lead to the reduction in *STM* transcript levels observed when silencing SnRK1 locally in the SAM. Similar conflicting results have also been reported for the SnRK1 animal homologue, AMPK, which despite being generally considered a growth repressor, is also essential for normal growth and development (González et al., 2020).

Further supporting this duality and the need to finely balance SnRK1 activity, defects in the SnRK1 myristoylation were previously associated to SAM function and plant development. The deficient myristoylation of the SnRK1β1 and β2 subunits in the N-myristoyltransferase mutant *nmt* was shown to contribute to SAM malformation and developmental arrest (Pierre et al., 2007). In the *nmt1-1* mutant, SnRK1 kinase activity and SnRK1β1 transcript levels were five-fold and two-fold higher, respectively, than in wild type plants. In the same study, GFP fusions of either SnRK1β1 or SnRK1β2 localized to the plasma membrane, whereas a G2A substitution preventing myristoylation of the β-subunits re-localized SnRK1β1 to the nucleus and SnRK1β2 to the cytoplasm, mimicking what happens in the *nmt1-1* line. It was hence suggested that myristoylation of β1 or β2 subunits sequester the

SnRK1 complex to the plasma membrane, acting as a negative regulator of SnRK1 signalling (Pierre et al., 2007). Thus, in this study, defects in SAM function were partly explained by an overactivation of SnRK1 signalling caused by defective myristoylation-mediated regulation.

Altogether, this highlights the importance of balancing SnRK1 activity: tipping the balance towards an overactivation or an inhibition disrupts the fine regulation of the meristem leading to various defects in STM expression and SAM function.

Future work using a line overexpressing SnRK1 locally in the SAM could provide complementary information on whether the decreased expression of STM-VENUS in the SnRK1α1 overexpressor plants is a consequence of the ubiquitous SnRK1 manipulation or is rather caused by a SnRK1 imbalance in the meristem. To further complement the local function established for SnRK1 in the SAM, it would be important to test how the amiRNA lines, where SnRK1 is disrupted locally in the SAM, behave when exposed to the different light and sugar treatments described in Chapter III. This could provide a more detailed knowledge on how STM could be differentially regulated by light and by different metabolic signals and at the same time, unveil the role of SnRK1 locally in this regulation. The versatility of these assays would also enable testing the impact of other factors such as glucose or auxin on the function of STM and other meristem regulators. All together, these results could complement what is known about STM regulation in the SAM and at the same time allow to disentangle the role of SnRK1 in the meristem by helping us understanding what are the factors modulating SnRK1 activity in the SAM and how these will impact key keepers of the SAM, such as STM.

Overall, this thesis brings novel insight into how environmental and metabolic signals converge into STM, a key keeper of the stem cell niche, potentially as a means to adjust SAM activity and plant growth and

development. Furthermore, this work uncovered the SnRK1 protein kinase as a key and multi-layered player in STM regulation, paving the key for more mechanistic studies in the future and for understanding the amazing developmental plasticity of plants.

5.4. References

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