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Metabolic regulation of the shoot apical meristem

by the TOR kinase in *Arabidopsis thaliana*

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Abbreviations

ABA	Abscisic acid
ADP	Adenosinediphosphate
AG	AGAMOUS
AHP6	ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERASE6
AHK	ARABIDOPSIS HISTIDINE KINASE
amiTOR	Artificial microRNA against TOR
AMP	Adenosinemonophosphate
ARR	ARABIDOPSIS RESPONSE REGULATOR
ATG	Autophagy
ATP	Adenosinetriphosphate
asTORis	Active site TOR inhibitory substance
6-BA	6-benzylaminopurine
CHX	Cycloheximide
CK	Cytokinin
CKX	CYTOKININ OXIDASE/ -DEHYDROGENASE
CRE1	CYTOKININ RESISTANT 1 allelic to AHK4
CYP735A	CYTOCHROME P450 735A
cZ	cis-Zeatin
CZ	Central zone
DEG	Differentially expressed gene
DNA	Deoxiribonucleic acid
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
GO	Gene ontology
GR	Glucocorticoid receptor

GUS	ß-glucuronidase
INCYDE	Inhibitor of cytokinin degradation
iP	isopentyladenine
iPR	isopentyladenosine
IPT	ISOPENTLYTRANSFERASE
LOG	LONELY GUY
NLS	Nuclear localization signal
mRNA	messenger Ribonucleic acid
OC	Organizing center
PUP	PURINE PERMEASE
PZ	Peripheral zone
RAPTOR	REGULATORY ASSOCIATED PROTEIN OF mTOR
RNAseq	Next generation RNA sequencing
RNA	Ribonucleic acid
ROP2	RHO OF PLANTS 2
SAM	Shoot apical meristem
S6K	P70 RIBOSOMAL S6 KINASE
TOR	TARGET OF RAPAMYCIN kinase
tRNA	transfer Ribonucleic acid
tZ	trans-Zeatin
tZR	trans-Zeatin riboside
uORF	Upstream open reading frame
UPR	Unfolded protein response
UTR	Untranslated region
WUS	WUSCHEL

Zusammenfassung

Pflanzen sind in der Lage ihre Entwicklungs- und Wachstumsprozesse dynamisch an sich verändernde Umweltbedingungen anzupassen, um ihre Aussichten auf erfolgreiche Reproduktion zu erhöhen. Diese bemerkenswerte Anpassungsfähigkeit basiert auf Stammzellen, die über den gesamten Lebenszyklus der Pflanze hinweg erhalten werden und so stetig neue Organe bilden. Um das Verhalten der Stammzellen an die jeweiligen Bedingungen anpassen zu können, haben Pflanzen ein hoch-spezialisiertes und komplexes Instrumentarium an Rezeptoren und Signalverarbeitungs- Netzwerken entwickelt. Folglich werden die zentralen molekularen Regelkreise, die für die Steuerung der Stammzellen verantwortlich sind an wechselnde Licht- und Nährstoffverfügbarkeit angepasst. Wir wissen, dass die Expression eines zentralen Steuerungsgens der Stammzellen, namentlich *WUSCHEL* (*WUS*), durch Photorezeptor- basierte Lichtsignale sowie Zucker- und Nitrat Verfügbarkeit moduliert wird. Um die Verarbeitung und Weiterleitung dieser Signalwege zu gewährleisten ist die Aktivität der evolutionär konservierten TOR Kinase notwendig. Bisher haben wir nicht verstanden, wie die Aktivität der TOR Kinase die Steuerung von *WUS* bewerkstelligt.

In der vorliegenden Arbeit gelang es mir basierend auf Transkriptom Untersuchungen das Pflanzenhormon Cytokinin als zentralen Effektor zwischen TOR Aktivität und *WUS* zu identifizieren. Ferner konnte ich zeigen, dass TOR Aktivität die Stabilität von *trans*-Zeatin bestimmt, ein Cytokinin welches bereits als eine der zentralen Determinanten des Sprosswachstums und der Meristem Steuerung identifiziert wurde. Zudem konnte ich die TOR abhängige translationale Unterdrückung von CKX katabolischen Enzymen als den zugrundeliegenden molekularen Mechanismus identifizieren. Dieser erlaubt es Pflanzen auf veränderte Umweltbedingungen zu reagieren, indem sie schnell die Verfügbarkeit von Wachstumshormonen begrenzen. Dies sind die ersten Untersuchungen die TOR abhängige translationale Unterdrückung von katabolischen Enzymen zeigen und tragen damit dazu bei, die spezifischen Einflüsse von zentralem Metabolismus und Entwicklungsgenen auf die Stammzellidentität in Einklang zu bringen.

Abstract

Plants dynamically adjust their development and growth pattern to maximize their chances to reproduce even under challenging conditions. The cellular basis for this remarkable phenotypic plasticity are stem cell populations that are maintained throughout the whole life of a plant. Intricate sensing and signaling mechanisms are required to instruct stem cell behavior according to current needs. Consequently, light- and nutrient signals are integrated and modulate the core molecular circuits underlying regulation of the shoot apical meristem (SAM). We know that expression of the stem cell master regulator *WUSCHEL* (*WUS*) is modulated by photoreceptor mediated light signaling pathways together with sugar and nitrate. Activity of the evolutionary conserved TOR kinase is required to integrate and relay these signals, but we do not understand how TOR activity is conveyed to modulate *WUS* expression.

In this work, I identified CK signaling in an RNAseq approach as the major downstream effector of TOR activity controlling *WUS* expression and shoot development. I demonstrate that TOR activity stabilizes *trans*-Zeatin which is known to be one of the major determinants influencing shoot growth and meristem maintenance. Mechanistically, this is achieved by TOR dependent translational repression of CKX catabolic enzymes, allowing plants to swiftly adjust their growth factor milieu in response to dynamic environments. This study is the first example for TOR mediated translational repression of a catabolic enzyme and thus provides a mechanistic framework integrating the contributions of central metabolism and core developmental regulators towards stemness.

Introduction

Plants are the main primary producers for organic compounds on our planet, they produce most of the atmospheric oxygen, they shape our landscapes and they constitute a major carbon sink. Despite their inability to move freely, land plants colonize the majority of the earth's landmass, well adapted to build up diverse ecosystems characterized by a wide spectrum of different soils, light- and climate regimes. Their ability to thrive in such a broad range of environments is undoubtfully based on their phenotypic plasticity. Unlike animals, plant development mainly occurs post-embryonically and plants can adapt organ numbers, -shape and -type but also chemical composition and growth rates according to the particular environment.

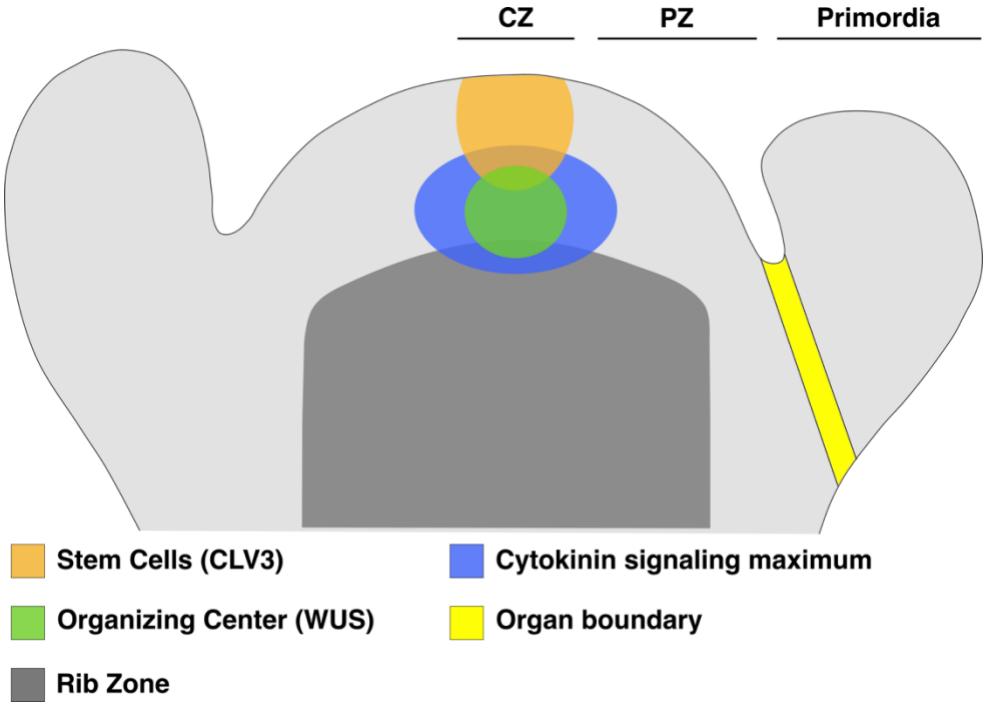
The cellular basis for this remarkable plasticity are so called meristems, which are pluripotent stem cell populations that are maintained throughout the whole life of a plant and enable them to grow new organs for thousands of years in extreme cases. In the plant embryo two primary meristems are initiated, namely the shoot apical meristem (SAM) and the root apical meristem (RAM) which are located at the shoot apex and the root apex respectively. Later during their lifecycle, plants can initiate further secondary meristems such as the cambium which is a radial stem cell population in the stem that enables secondary growth and is responsible for wood formation. Axillary meristems are also initiated post-embryonically to grow secondary shoot branches, similarly to lateral root meristems that build up the branched root systems of plants. Additionally, plants can initiate *de novo* meristems during regeneration in order to regrow a new plant from minute amounts of damaged tissue. Furthermore, meristems can change their identity, for example during the transition from vegetative- to reproductive development, when the primary SAM is reprogrammed in order to produce flowers and eventually terminates.

Meristem structure and activity not only determine when and where new organs are produced but also how these organs will look like and which identity they will acquire. The primary SAM after being initiated in the embryo, first enters a dormant stage where no organs are produced until the seed germinates and perceives light, whereupon the SAM becomes active to produce true leaves. Subsequently, environmental factors such as daylength, light quality and temperature instruct the SAM to first produce sepals, then petals, then stamen and finally a gynoecium. Depending on the species, the number and relative position of these organs can be quite variable. The developmental history of a single SAM exemplifies that intricate mechanisms are required to control when and which organ is produced.

As plants are facing constant competition for resources in their habitats, maximizing resource use efficiency is crucial to enable ecological success of individuals and eventually of whole species. Therefore, optimal control of meristem activity and ideal timings of developmental transitions lay the basis for a successful ecological competition, as meristems constitute major sinks for resources. It is hence not surprising that numerous research efforts try to decode the molecular mechanisms underlying meristematic control. This will not only deepen our understanding of plant function and evolution but hopefully also facilitate breeding strategies that might contribute to a more productive and more sustainable agriculture.

Meristem structure

Meristems are polarly organized tissues, forming an almost linear continuum from undifferentiated stem cells towards progressively more differentiated cells. One pole comprises the so-called stem cell niche or organizing center, which instructs adjacent cells to acquire stem cell fate thereby preventing their differentiation. These cells divide asymmetrically in order to self-renew and maintain stemness and at the same time secrete daughter cells away from the niche, which become transit amplifying cells characterized by high proliferation rates, forming the basis for the emerging organs. This polarized niche concept can be observed not only in plant meristems but is found also in animal stem cell systems (Heidstra & Sabatini, 2014).



Scheme1: Organization of the shoot apical meristem Schematic representation of the SAM depicting the most relevant functional domains and hormone signaling environment. Adapted from Janocha et al. 2018

In the SAM the stem cell niche is located in the central zone (CZ) of the dome shaped tissue, harboring stem cells in the three uppermost cell layers (L1-L3) and the organizing center (OC) right below the stem cell population (Gaillochet et al., 2015). From the CZ cells are secreted either towards the basis where they form the rib meristem that generates the plant vasculature and the stem, or towards the peripheral zone (PZ) of the SAM where cells amplify and eventually organ primordia are initiated. The rate at which cells are produced in the CZ, the rate at which they transition towards the periphery and the rate at which organs are formed has to be tightly coordinated between all parts of the tissue, as imbalances might deplete the stem cell pool or prevent organogenesis. This is particularly challenging for plant cells as they are physically constraint by a rigid cell wall rendering them immobile, wherefore intricate sensing and coordination mechanisms have to ensure tissue integrity throughout continuous division and growth.

Molecular control

On the molecular level the OC is characterized by the expression of WUSCHEL (WUS), which is the central element of the molecular module maintaining stem cells of the SAM (Mayer et al., 1998; Schoof et al., 2000). WUS is a homeodomain transcription factor that acts non-cell-autonomously, as it moves from the OC where it is expressed through cytoplasmic channels towards the apical stem cells, preventing their differentiation (Daum et al., 2014). Concomitantly, WUS induces expression of CLAVATA3 (CLV3) which is processed towards a secreted peptide that restricts *WUS* expression in a paracrine fashion, acting through several receptor modules expressed in the meristem such as CLAVATA1 (CLV1), CLAVATA2 (CLV2), CORYNNE (CRN), RECEPTOR-LIKE-PROTEIN KINASE 2 (RPK2) and BARELY ANY MERISTEM (BAM) (Hazak & Hardtke, 2016). The negative feedback loop between WUS and CLV3 comprises the core regulatory module for stem cell maintenance, as loss of either of those two functions leads to either termination of the SAM or massive stem cell over-proliferation and hence to severe developmental defects (Mayer et al., 1998; Schoof et al., 2000). In the current dogma, WUS is perceived as a transcriptional master regulator of stem cells and many interactions influencing stem cell maintenance or activity are thought to modulate WUS function.

Despite its very pronounced role in stem cell regulation, there is relatively little information about WUS´s molecular functionality. One early identified direct target gene of WUS is AGAMOUS (AG) which is an important regulator of flower development (Lenhard et al., 2001). However, WUS controls AG expression only in the context of the floral meristem but

not in the vegetative SAM, wherefore it is thought that WUS requires cofactors conferring target gene specificity and consequently LEAFY (LFY) has been identified to mediate WUS control of AG expression (Lohmann et al., 2001). Recent findings corroborate the hypothesis of WUS requiring cofactors, as its homeodomain possesses a relatively low affinity towards DNA motifs bound by WUS compared to other homeodomains (Sloan et al., 2020). In addition, WUS has been shown to repress transcription of *type-A ARRs* (*ARR5, ARR6, ARR7, ARR15*) (Leibfried et al., 2005), which are negative regulators of cytokinin signaling, causing a smaller SAM with fewer stem cells if not repressed by WUS. It thus appears that a major function of WUS is to establish and maintain hormonal balance in the CZ of the SAM that prevents differentiation. This notion is strongly supported by the recent finding that WUS acts as "molecular rheostat" for phyto-hormone signaling in the SAM, where it inhibits auxin signaling, thereby preventing differentiation in the CZ while at the same time ensuring a minimal level of auxin signaling that appears necessary to maintain stem cells (Ma et al., 2019). The same study suggests that WUS binds to a whole batterie of promoters controlling the expression of auxin pathway members, putatively by recruiting histone de-acetylases (HDACs) to the respective target loci, which results in dampened expression levels and eventually in a low auxin signaling micro-environment.

Plant hormones in the SAM

Many processes in plants are controlled by phytohormones which are a diverse group of potent signaling molecules that can have very different and sometimes even contrary effects depending on the cellular and developmental context. In the SAM several plant hormones have been reported to control numerous aspects of cellular growth dynamics and cellular interactions that eventually determine the properties of the SAM and shape plant morphogenesis. As already indicated, the SAM is subdivided into functionally distinct domains which often correlate with a specific hormone signaling regime. Most prominent are the maxima of auxin signaling in the PZ marking the site of organ primordia initiation and consequently, ectopic application of auxin can initiate primordia emergence (Reinhardt et al., 2000, 2003). In contrast, the CZ is characterized by low levels of auxin signaling as mentioned in the previous section (Ma et al., 2019). Thereby, the local abundance of the hormone is a major factor determining the output of the respective signaling pathway. Local hormone homeostasis is a result of biosynthesis, distribution and turnover of the hormone and the effects of disturbed homeostasis can be drastic. This is exemplified by the phenotype of *null* mutants of the auxin transporter *pin1*, which lack the ability to properly distribute auxin and as a consequence do not produce any flower primordia but instead have a "pin shaped"

inflorescence (Gälweiler et al., 1998). However, hormonal imbalance will often result in altered properties of the SAM regardless of where it is interfered with. The importance of plant hormones for plant stem cells cannot be stressed enough as already the initiation of the stem cell niche is governed by an interplay between auxin and cytokinin (CK) during early embryogenesis (Zhang et al., 2017) and can be recapitulated during shoot regeneration experiments from tissue culture where specific ratios of auxin and CK are supplied in the culture medium in order to initiate *de novo* meristem formation (Pernisova et al., 2018). The effect of hormones is conveyed by signaling cascades that eventually lead to transcriptional changes (Blázquez et al., 2020; Vanstraelen & Benková, 2012). Each hormone signaling pathway is characterized by a unique set of receptors and signal transduction machinery that, after sensing the presence of the respective hormone, can initiate a signaling cascade that transmits a signal to the nucleus where transcriptional regulators are activated or inhibited. Many of the major hormone signaling pathways have central functions regulating different aspects of SAM homeostasis. Giberellic acid (GA) regulates expression of the floral commitment gene APETALA1 through DELLA proteins, thereby controlling the transition of the SAM from vegetative to reproductive development (Yamaguchi et al., 2014). Similarly, brassinosteroids (BR) regulate tissue architecture of the SAM by controlling the transcription of CUP-SHAPED COTYLEDON (CUC) genes that establish organ boundaries (Gendron et al., 2012). Even though each hormone has its unique signaling cascade, extensive crosstalk between many hormone signaling pathways exists and can occur at multiple levels. Often, one pathway controls transcription of a negative regulator of another pathway and thereby generates a specific hormone signaling environment or patterning. Along this notion, CK induces transcription of GA catabolic enzymes to generate a low GA signaling environment in the SAM to prevent differentiation in the center (Jasinski et al., 2005). Similarly, auxin in order to warrant robust patterning of organ initiation in the SAM induces transcription of ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERASE 6 (AHP6), a negative regulator of cytokinin signaling and thereby provides an inhibitory field that regulates the pace at which new organs are initiated and thus determines phyllotactic pattern (Besnard et al., 2014). Alternatively, pathways can also converge on shared signaling components, as is the case for Ethylene and CK, where the histidine kinase receptors of both pathways are able to induce a signaling cascade, phosphorylating the same AHPs and type-B ARABIDOPSIS RESPONSE REGULATOR (ARR) transcriptional activators (Binder et al., 2018).

Cytokinin

Although many hormones control specific aspects of SAM regulation, CK deserves specific attention as it is a key regulator of SAM homeostasis especially in the CZ of the SAM where it is thought to control the domain architecture and to determine the relative position of the stem cell niche (Chickarmane et al., 2012; Gordon et al., 2009; Gruel et al., 2016). Moreover, cytokinin has been shown to be the main driver of *WUS* expression and in shoot regeneration experiments CK controls the transition from root to shoot identity whereas it can be considered a shoot identity factor (Pernisova et al., 2018). CK also fuels shoot growth and development in response to the environment as different nitrate concentrations in the soil result in altered CK biosynthesis rates and consequently modulate CK signaling and *WUS* expression in the SAM which ultimately modulates growth (Landrein et al., 2018; Takei et al., 2004). Light is another environmental factor determining CK signaling strength in the SAM thereby modulating stem cell activity and growth pattern (Pfeiffer et al., 2016; Yoshida et al., 2011). CK also plays an important role in plants response towards several stress conditions such as drought or heat (Huang et al., 2018; Prerostova et al., 2018). All these examples demonstrate the central role of CK for SAM homeostasis during regular maintenance but also show that CK is a central hormone mediating stem cell adaptation to the environment. Therefore, a complete understanding of CK signaling and homeostasis is crucial to understand how stem cells are maintained and how they adapt to dynamic environments.

Cytokinin metabolism and transport

CK is an adenine derived compound that is conjugated at the N^6 position with an isoprenoid side chain, where the side chain structure and its biological origin can be variable (Reviews (Kieber & Schaller, 2014; Sakakibara, 2010)). The side chain determines the biological activity of the respective compound. CK metabolites can be present in their free base form or conjugated either to a ribotide sugar or glucose at different positions of their adenine core. In Arabidopsis the most active CKs are the free bases of *iso*pentyladenine (*i*P), Dihydrozeatin (DZ) and the most abundant CK *trans*-Zeatin (*t*Z). While these CKs are all synthesized from ATP, ADP or AMP, the fourth class of CKs *cis*-Zeatin (*c*Z) is thought to be derived from the brake down of isopentylated tRNA molecules and does not possess strong CK activity in classical bioassays in Arabidopsis (Gajdošová et al., 2011).

The initial step in CK biosynthesis is the addition of the isoprenoid side chain to ATP, ADP or AMP and is catalyzed by ISOPENTYLTRANSFERASEs (IPTs) (Takei et al., 2001). The side chain precursor is Dimethylallylpyrophosphat (DMAPP) that is supplied either from the

chloroplastic methylerythritol phosphate (MEP) pathway or from the cytosolic mevalonate (MVA) pathway, although it appears that the MEP pathway is the major contributor for CK synthesis (Kasahara et al., 2004). The initial products of this reaction are either *iso*pentyladenosine-5´-triphosphate (*i*PRTP), *iso*pentyladenosine-5´-diphosphate (*i*PRDP) or *iso*pentyladenosine-5´-monophosphate (*i*PRMP) which then serve as substrates for side chain hydroxylation to produce *trans*-Zeatin riboside 5´-mono(di-, tri-)phosphate (*t*ZRMP). The conversion of *i*PRMP to *t*ZRMP is catalyzed by the cytochrome P450 isoforms CYP735A1 and CYP735A2 (Takei et al., 2004). In Arabidopsis nine members of the IPT gene family have been identified which all have specific expression pattern and distinct subcellular localization either in chloroplasts, in mitochondria or the cytosol (Takei et al., 2001). Most IPTs synthesize *i*P and only IPT2 and -9 are thought to mediate isopentylation of tRNAs from which *c*Z is derived (Gajdošová et al., 2011).

To obtain the active free bases, the ribotide precursors need to be further processed by so called LONELY GUY (LOG) enzymes. There are seven LOG isoforms in Arabidopsis and disrupting their function can have severe consequences for root and shoot development, as particularly LOG4 that is expressed in the L1 layer of the SAM seems to be vital for proper stem cell homeostasis (Chickarmane et al., 2012; Gruel et al., 2016; Kuroha et al., 2009; Tokunaga et al., 2012).

CK levels are not only regulated at the biosynthesis level but can also be inactivated by conjugation to a glucose moiety, either at the N^7 or N^9 position of their adenine core, or at the oxygen in the side chain of *t*Z and *D*HZ. N- and O-glycosylation are catalyzed by different glycosyltransferases and while O-glycosylation by UGT85A1 can be reversed by β-glucosidases, N-glycosylation by either UGT76C1 or UGT76C2 is thought to be irreversible (Brzobohaty et al., 1993; Hou et al., 2004; Jin et al., 2013).

Due to their nature as signaling compounds plants require cellular- and systemic distribution systems for CK. However, our understanding of CK distribution and the underlying transport systems is rather scarce. *t*Z is predominantly synthesized in roots particularly when soil nitrate levels are high and transported to aerial tissues mainly as *t*ZR via the xylem sap, while *i*P is thought to be transported via the phloem from shoot to root (Kudo et al., 2010; Landrein et al., 2018; Matsumoto-Kitano et al., 2008; Osugi et al., 2017). Only one exporter for CK has been identified to date which is a member of the ATP-binding cassette transporter subfamily G14 (ABCG14) and is thought to translocate CKs to the xylem (Ko et al., 2014). For cellular import, members of the PURINE PERMEASE (PUP) and equilibrative nucleoside

transporter (ENT) families have been identified to possess CK transport capacity (Kudo et al., 2010). However, only PUP14 has been characterized in more detail and was shown to possess huge potential to sequester active CKs away from the apoplast resulting in decreased CK signaling output. This observation has led to a change in the former dogma that CKs are perceived only at the ER membrane (Antoniadi et al., 2020; Zürcher et al., 2016).

Cytokinin catabolism

One class of very potent enzymes controlling CK homeostasis are CYTOKININ OXIDASES/ DEHYDROGENASES (CKXs), catalyzing the irreversible cleavage of the side chain of many CK species. There are seven CKXs encoded in the Arabidopsis genome which all differ in their expression pattern, subcellular localization, substrate specificity and pH optimum (Frébortová et al., 2004; Galuszka et al., 2005, 2007; Kowalska et al., 2010; Werner et al., 2003). Except CKX7 (Köllmer et al., 2014), all CKXs possess a signal peptide for the secretory pathway at their N-terminus and some are predicted to be secreted to the apoplast, while others have been shown to remain in the ER lumen or are integral ER membrane proteins (Bilyeu et al., 2001; Galuszka et al., 2005; Niemann et al., 2018; Werner et al., 2003). However, in Arabidopsis only CKX1 localization has been thoroughly characterized as an integral ER membrane protein. Investigation of the endogenous CKX proteins is still missing and remains a challenge due to the relatively low expression and protein levels. The potency of this enzyme class is underpinned by the severity of their overexpression phenotypes. Global overexpression often causes severely delayed and stunted shoot development while at the same time enhancing root growth (Bartrina et al., 2011; Holst et al., 2011; Werner et al., 2001, 2003, 2008). Moreover, agronomically important traits like resistance to several stresses or fruit- and organ size and seed yield can be controlled by targeted expression of CKX enzymes, rendering them interesting targets for crop improvement (Bartrina et al., 2011; Werner et al., 2003, 2010). Reduced CKX activity often leads to increased SAM size and enhances expression of *WUS* (Bartrina et al., 2011; Pfeiffer et al., 2016; Werner et al., 2001, 2003). Interestingly, the expression domain of *CKX3* is congruent with the *WUS* expression domain further highlighting the importance of CKXs for stem cell regulation (Bartrina et al., 2011). Together, these observations point towards a role of CK and CKXs in the control of sink strength of a particular tissue, thereby determining the amount of resources that are allocated to a particular organ. This makes CKXs interesting candidates regarding environmental adaptation, because they were found to be transcriptionally regulated by light, nutrients or biotic and abiotic stresses (Carabelli et al., 2007; Werner et al., 2006). However, our understanding of CK metabolism can be still considered rudimentary, especially regarding tissue- but also subcellular

distribution of CK metabolites. The presence of biosynthetic, catabolic and signaling components in almost all organelles and the apoplast imply a very intricate interplay between the control of local CK availability and the CK signaling apparatus.

Cytokinin signaling

Perception of CKs is mediated by a two-component system similar to that of bacteria. In Arabidopsis three ARABIDOPSIS HISTIDINE KINASE (AHK) receptors have been identified, namely AHK2, AHK3 and AHK4 (allelic to CRE1 and WOL1) (Lomin et al., 2011, 2012). The receptors are composed of an extracellular CHASE (Cyclase/Histidine kinase Associated Sensory Extracellular) domain that binds to CK and initiates autophosphorylation of the kinase- and the receiver domain, thereby initiating a signaling cascade which involves phosphorylation of ARABIDOPSIS HISTIDINE PHOSPHOTRANSFERASEs (AHPs) that shuttle into the nucleus where they pass on the phosphoryl group to ARABIDOPSIS RESPONSE REGULATORs (ARRs) that then act as transcriptional activators of target gene expression (Müller, 2011). Among their primary targets are type-A ARRs that serve as negative regulators of CK signaling, thereby modulating CK response (Hwang & Sheen, 2001; To et al., 2007). Each step of the multistep phosphorelay process is encoded by multiple family members and single knock out mutants mostly have no or only relatively mild phenotypes, which points towards a high redundancy in gene function (Müller, 2011). However, some degree of specificity is generated by specific expression profiles of different isoforms. Furthermore, in addition to their very distinct expression domains in roots and shoots the AHK receptors differ in their affinities towards different active CK ligands (Romanov & Lomin, 2006; Spíchal et al., 2004; Yonekura-Sakakibara et al., 2004). Consequently, different double mutant combinations result in distinct root and shoot specific phenotypes (Gordon et al., 2009; Lomin et al., 2012; Riefler et al., 2006).

Six family members of the AHPs (AHP1-6) with largely redundant function mediate the phosphoryl transfer from the cytosol to the nucleus (Hutchison et al., 2006; Suzuki et al., 2000). While most AHPs serve as positive regulators of CK signaling, AHP6 lacks a conserved His-residue rendering it a pseudo-phosphotransferase that inhibits the phophorelay initiated by CK. AHP6 action is particularly important in the context of vascular development in the root and during the establishment of phyllotactic pattern in the SAM (Besnard et al., 2014; Mähönen et al., 2006). Interestingly, AHPs are also dephosphorylated by AHK4, which in addition to its kinase function also possesses phosphatase activity that is repressed once CK is bound (Mähönen et al., 2006). The AHPs are thought to shuttle between the cytosol and nucleus

where they phosphorylate type-B ARR transcription factors that activate target gene expression (Punwani et al., 2010).

Eleven type-B ARRs mediate transcriptional activation of CK targets which act partly redundant but also seem to have specific functions in multiple contexts such as leaf differentiation, leaf senescence, proliferation or regeneration (Hwang & Sheen, 2001; Meng et al., 2017; Xie et al., 2018; Zhang et al., 2017). Numerous gene regulatory regions are bound by type-B ARRs involved in a myriad of functions. However, recent studies show that type-B ARR targets are specifically enriched in genes involved in the regulation of other hormone signaling pathways (Xie et al., 2018; Zubo et al., 2017), which could mean that they readjust hormonal balance in response to external stimuli. Noteworthy, type-B ARRs seem not only to act as transcriptional activators but also pioneer chromatin remodeling controlling chromatin accessibility and histone modifications of target loci (Potter et al., 2018; Zhang et al., 2017). In addition, type-B ARRs are crucial for SAM regulation as several of them were shown to control *WUS* transcription by direct binding to its promoter (Meng et al., 2017; Sakai et al., 2001; Xie et al., 2018; Zhang et al., 2017; Zubo et al., 2017).

One important class of direct CK target genes are type-A ARRs that are induced within minutes upon CK perception and act as negative regulators of CK signaling. As such type-A ARRs serve as important modulators of CK homeostasis particularly in the context of SAM regulation and due to their nature as primary targets, their expression is widely used as a read out for CK pathway activation (Buechel et al., 2010; D'Agostino et al., 2000; Hwang & Sheen, 2001; Leibfried et al., 2005; Zhao et al., 2010).

The severe effects of altered CK homeostasis and -signaling on plant development and -physiology underpin the importance of understanding how CK signaling is controlled, as modulation of the CK pathway plays a central role in plants adaptation to environmental change (Müller et al., 2015; Takei et al., 2004). As such CKs relay information about the light regime and soil nitrate levels towards the SAM thereby controlling stem cell activity (Landrein et al., 2018; Osugi et al., 2017; Pfeiffer et al., 2016).

Environmental adaptation

As sessile organisms, plants lack the ability to mitigate detrimental environments and therefore evolved alternative strategies to cope with stressful conditions. Consequently, plants produce secondary metabolites to shield themselves from biotic and abiotic stresses but also specifically adapt their growth pattern and developmental transitions to meet the requirements of a particular environment. This complex and multifaceted process requires constant surveillance

of external and internal factors, wherefore a significant part of the plant genome codes for receptors and other signaling components. This is exemplified by the more than 600 receptor-like kinases encoded in the Arabidopsis genome (Shiu et al., 2004). However, the myriad of signals originating from the sensory apparatus must be processed and integrated to generate an adequate cellular and physiological response. Our current understanding of signal integration is relatively scarce, but a few molecular nodes have been identified and give us a glimpse of how, diverse and/ or contrary signals might be integrated and translated into a tailored cellular- and ultimately organismal response. Signal integration can occur on all levels of the signaling process (Janocha & Lohmann, 2018). Receptors compete for a required co-receptor, kinases and phosphates exert antagonistic effects on a specific phospho-site and transcription factors compete for specific binding motifs in the genome. In the context of plant stem cell regulation, the TARGET OF RAPAMYCIN (TOR) kinase network has recently emerged as an integrator of photoreceptor mediated light signaling pathways and the endogenous sugar status in order to control promoter activity of *WUS* in the SAM and hence leaf organogenesis at the photomorphogenic transition (Pfeiffer et al., 2016).

The TOR kinase network

TOR was named after the substance rapamycin which was originally isolated from the soil microbe *Streptomyces hygroscopicus*. This has been found in soil samples from Rapa Nui after which the substance was named and the circumstances of its discovery are a worthy lecture themselves (Seto, 2012). The TOR complex and the TOR kinase comprising its catalytic core have been identified in yeast and mammals due to their binding with the FKBP12-rapamycin complex that inhibits TOR kinase activity and builds the basis for the initially observed anti-fungal, anti-tumoral and immunosuppressive effects of rapamycin. TOR is a PI3K serine/ threonine kinase that is conserved in all eukaryotes and in mammals two functionally different TOR complexes exist that are defined by a distinct subunit composition. FKBP12 is only part of mTORC1 but not mTORC2, which is why only mTORC1 is sensitive to rapamycin treatment. Only mammalian lethal with SEC13 protein 8 (mLST8) and DEP-domain-containing mTOR interacting protein (DEPTOR) are found in both complexes, while the regulatory associated protein of mTOR (RAPTOR) and proline-rich AKT substrate 40kDa (PRAS40) are found exclusively in mTORC1, while the proteins RICTOR, protein associated with rictor 1 or 2 (PROTOR1/2) and MAPK-interacting protein 1 (mSIN1) define mTORC2. In contrast, only homologs for FKBP12, LST8 and RAPTOR have been identified in plants which is why currently it is thought that only a single TOR complex exists in plants, however scarce evidence suggests that additional complexes might also be present in plants. mTORC1

has been characterized to a much greater extent than mTORC2 which is why most of the mentioned studies refer to mTORC1 only.

In all organisms that possess a TOR homolog, its activity promotes cellular growth and proliferation. TOR therefore reads out the nutritional state of a cell and promotes anabolic processes that are required to fuel growth and proliferation, while it appears to restrict catabolic turnover of macromolecules and organelles predominantly through inhibition of autophagosome formation. Most prominently, TOR promotes translation by phosphorylation of p70 S6 kinase 1 (S6K1) which in turn phosphorylates ribosomal protein S6 (RPS6) a central regulator of ribosome biogenesis, tRNA transcription and translational elongation (Chauvin et al., 2014; Mayer et al., 2004; Shor et al., 2010). This central signaling axis appears to be well conserved in plants and in many other eukaryotes and in plants this axis controls translation re-initiation rates of specific uORF containing transcripts (Brunkard, 2020; Dobrenel et al., 2016; Ren et al., 2011; Schepetilnikov et al., 2013; Xiong et al., 2013; Xiong & Sheen, 2012). TOR also phosphorylates eukaryotic translation initiation factor 4E-binding proteins (4E-BPs) thereby globally increasing 5´cap-dependent translation (Hara et al., 1997). In plants, no homolog of 4E-BP has been identified to date and it is not clear whether a similar mechanism acts in plants. In addition, TOR regulates many players of central metabolism such as ATF4, SREBP1/2 and HIF1α, controlling nucleotide-, lipid biosynthesis and glycolysis respectively (Ben-Sahra et al., 2016; Düvel et al., 2010; Horton et al., 2002). Complementing its function in promoting anabolism, TOR prevents the formation of the autophagosome by phosphorylation of unc-51-like autophagy-activating kinases 1 (ULK1) and ATG13, thereby limiting catabolic turnover of organelles and macromolecules (Hosokawa et al., 2009; Kim et al., 2011). Many components of the autophagosome are conserved in plants and their assembly in part depends on TOR activity. Just recently proof for direct phosphorylation of ATG components by TOR has been found (Soto-Burgos & Bassham, 2017; Suttangkakul et al., 2011). The role of TOR as a negative regulator of catabolism is underpinned by its inhibition of lysosome biogenesis and its localization at lysosomal membranes (Demetriades et al., 2014; Menon et al., 2014). However, the localization of TOR is still a controversial topic as besides its lysosomal localization there exists evidence for localization in the cytosol, the ER membrane, the vacuole, the peroxisome, mitochondria, the plasma membrane or the nucleus, although its function in the respective organelles is not always clear (Betz & Hall, 2013). In plants there is limited evidence for TOR localization at endosomal membranes (Schepetilnikov et al., 2017). The second complex mTORC2 is not sensitive to rapamycin and is involved in rearrangement of the cytoskeleton, cell mobility and membrane curvature (Fu & Hall, 2020).

As central hub controlling energy intensive anabolic processes TOR activity is modulated by several pathways sensing the nutrient and energy status of the cell. Particularly, amino acid availability affects TOR activity through the GTPases Rag and Rheb and its recruitment to lysosomes (Demetriades et al., 2014; Kim et al., 2008; Sancak et al., 2008). The growth factor insulin is another important activator and acts through inhibition of the TOR repressor PRAS40 (Garami et al., 2003; Sancak et al., 2007). Other TOR antagonists such as AMPK or the TSC complex are repressed when sufficient energy is available but can be activated during hypoxic conditions or starvation regimes (DeYoung et al., 2008; Gwinn et al., 2008).

In plants, TOR appears to have adapted to the specific requirements of photoautotrophs, as one of the major determinants of TOR activity seems to be light availability. Interestingly, this is not limited to the availability of photoassimilates but also requires the input of photoreceptor mediated light signaling pathways mediated by the E3 ligase CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) (Li et al., 2017; Pfeiffer et al., 2016). Consequently, the essential macronutrients nitrogen and sulfur but also other plant specific compounds such as auxin and glucosinolates act as modulators of TOR activity (Dong et al., 2017; Liu et al., 2021; Malinovsky et al., 2017). Auxin is thought to mediate the effect of light downstream of COP1 and was shown to activate TOR via rho of plants 2 (ROP2), which also mediates the effect of different nitrogen sources on TOR by direct phosphorylation of the Serine2424 (S2424) (Li et al., 2017; Schepetilnikov et al., 2017). TOR activity depends on complex formation with RAPTOR and can be dissociated by phosphorylation of the latter. The plant homolog of the yeast AMPK sucrose non-fermenting-1 related protein kinase 1 (SnRK1), but also members of the SnRK2 family have been demonstrated to negatively affect complex formation between TOR and RAPTOR in response to energy deprivation or the stress hormone abscisic acid (ABA) (Nukarinen et al., 2016; Wang et al., 2018). In turn, under favorable conditions TOR activity inhibits SnRK2 activation through phosphorylation of the Pyrabactin Resistance 1–Like (PYL) ABA receptors (Wang et al., 2018). This reciprocal mechanism enables the continuous integration of growth promoting and -repressing processes similarly to a rheostat function, contrasting with an on- or off switch behavior that would not allow finely tuned responses.

As in other organisms, plant TOR functions in growth control and as indicated earlier, enables anabolic processes required for growth but also leads to adaptation of primary metabolism to meet the needs of the current growth regime. Consequently, TOR activity is required to grant nutrient utilization as inactivation leads to the accumulation of storage lipids, starch and free amino acids (Caldana et al., 2013). Recently a role of TOR in the regulation of plasmodesmata

has been identified, further highlighting the adaptation of TOR function to the requirements of plants (Brunkard et al., 2020). In line with the perception of TOR as a growth regulator it was shown to control central aspects of meristem function and maintenance. Initially, it was shown that size and proliferative activity of the RAM depend on TOR activity in response to glucose or other energy rich metabolites which is partially mediated by direct phosphorylation of the cell cycle regulator e2Fa (Van Leene et al., 2019; Xiong et al., 2013). In the SAM not only proliferation rates and growth depend on TOR being active but also the expression of *WUS*. This was first described on the transition from skotomorphogenic- to photomorphogenic development, led by the observation of *WUS* not being expressed in etiolated seedlings and only becoming active when either light is perceived or sugars are exogenously supplied (Chen et al., 2018; Pfeiffer et al., 2016). While both stimuli independently induce basal expression of *WUS*, synergistic activation leads to additive expression levels and only then organogenesis of true leaves is initiated. Interestingly, sugar- but also light signaling mediated *WUS* induction depends on active TOR rendering it a central gate keeper for SAM activation. Other studies found, that early primordia development also depends on light mediated TOR signaling and that auxin mediates the light dependent effects by activating ROP2 (Li et al., 2017). Only recently it was discovered that TOR phosphorylates EIN2 a transcriptional master regulator in the context of glucose dependent hypocotyl growth in etiolated seedlings and that a major proportion of the transcriptomic changes induced by the glucose-TOR signaling axis are governed *via* this transcription factor (Fu et al., 2021). Although this study exemplifies how large-scale transcriptional rearrangements in response to altered TOR signaling can be conveyed, the relevance of these findings for photomorphogenic plants and other tissues is rather limited.

Aims of this study

Numerous studies have connected TOR to many different cellular and physiological processes and several direct phosphorylation targets and interactors has been identified. However, it remains unclear how TOR exerts its diverse functions especially in the context of stem cell control and meristem maintenance and particularly how TOR controls stem cells in the SAM and *WUS* expression is unknown. Thus, this study aims to elucidate the role of TOR in stem cell control in the SAM and to identify downstream mediators that relay TOR activity to control expression of *WUS*. Moreover, this study tries to improve our general understanding of TOR function in plants.

Materials

Most experiments have already been described in (Janocha et al., 2021) and have been copied and modified from there.

Plants

All used plant lines were in the Col-0 background. The double reporter line pWUS:3xVenus:NLS/ pCLV3:mCherry:NLS, the pTCSn:GUS line, as well as the *ckx5*, *ckx6* and *ckx5/ckx6* CRISPR mutants are described in (Pfeiffer et al., 2016). The p35S:ARR1ΔDDK:GR line is described in (Sakai et al., 2001) and was crossed with the double reporter line to obtain homozygous alleles for each transgene. The p35S:cMyc-CKX1 line is described in (Niemann et al., 2015, 2018). The *ckx2* (SALK_083761c), *ckx3* (SALK_050938c), *ckx4* (SALK_055204c) mutants were obtained from NASC. The *cre1-2/ahk3-7* mutant was described in (Riefler et al., 2006). The *rop2-1* mutant was obtained from NASC (SALK_055328C). The lines expressing wild type and dominant negative alleles of ROP2 (DN-ROP2) are described in (Denninger et al., 2019). The *raptor1b* mutant has been obtained from NASC (SALK_078159). The pAHP6:GFP line is described in (Mähönen et al., 2006) and the pAHP6:AHP6-Venus line is described in (Besnard et al., 2014). The p35S:GFP-ATG8 line and the atg5xp35S:GFP-ATG8 line are described in (Dauphinee et al., 2019).

Antibodies

Primary antibodies

Anti-GFP (ab1218)	abcam; Cambridge, UK
Anti-pS6K1(pT449)	abcam; Cambridge, UK
Anti-S6K1/2 (AS12-1855)	Agrisera AB; Vännäs, Sweden
Anti-cMyc (9E10)	Santa Cruz Biotechnology; Dallas, TX

Secondary antibodies

Anti-mouse IgG-HRP (sc-2318)	Santa Cruz Biotechnology; Dallas, TX
Anti-rabbit IgG-HRP (sc-2357)	Santa Cruz Biotechnology; Dallas, TX

Plant treatments

DMSO	Roth; Karlsruhe, Germany
AZD8055	Selleckchem; Houston, TX

TORIN1	Selleckchem; Houston, TX
KU638794	Selleckchem; Houston, TX
6-Benzylaminopurine	Merck; Darmstadt, Germany
*Iso*pentyladenine	Duchefa; Haarlem, The Netherlands
*Iso*pentyladenosine	Duchefa; Haarlem, The Netherlands
trans-Zeatin	Duchefa; Haarlem, The Netherlands
trans-Zeatin riboside	Duchefa; Haarlem, The Netherlands
cis-Zeatin	OlChemim; Olomouc, CZ
cis-Zeatin riboside	OlChemim; Olomouc, CZ
Kinetin	Duchefa; Haarlem, The Netherlands
Cycloheximide	Merck; Darmstadt, Germany

GUS staining

Potassium Ferrocyanide (K-Ferro)	Merck; Darmstadt, Germany
Potassium Ferricyanide (K-Ferri)	Merck; Darmstadt, Germany
X-Gluc	Roth; Karlsruhe, Germany
PBS	Roth; Karlsruhe, Germany

Plant growth

Murashige and Skoog salts	Duchefa; Harlem, Netherlands
Phyto-agar	Duchefa; Harlem, Netherlands
Silwet L-77	Lehle Seeds; Round Rock, USA
Triton-X100	Roth; Karlsruhe, Germany
Square petri dishes	Greiner; Frickenhausen, Germany
Nylon mesh nitex 03/100-44	Sefar; Heiden, Switzerland

RNA-extraction, cDNA synthesis, qRT-PCR

Plant RNA Purification Reagent	Invitrogen; Carlsbad, CA
RNAeasy plant mini kit	Qiagen;Hilden,Germany
TURBO DNAse Ambion/	Thermo Fisher; Waltham, MA
RevertAid First Strand cDNA synthesis kit	ThermoScientific; Waltham, USA
SG qPCR Master Mix	EURx; Gdansk, Poland

Western blot

MOPS buffer	Roth; Karlsruhe, Germany

ß-Mercapthoethanol	Roth; Karlsruhe, Germany
Glycine	Roth; Karlsruhe, Germany
SDS	Roth; Karlsruhe, Germany
EDTA	Roth; Karlsruhe, Germany
AEBSF	Roth; Karlsruhe, Germany
Protease inhibitor cocktail	Merck; Darmstadt, Germany
BSA	Roth; Karlsruhe, Germany
Immun-Blot PVDF	Bio-Rad laboratories; USA
ECL Select	Cytiva; Amersham, UK
Acrylamide	Roth; Karlsruhe, Germany
TEMED	Roth; Karlsruhe, Germany
Tween 20	Roth; Karlsruhe, Germany
Amidoschwarz	Roth; Karlsruhe, Germany

Plant culture media

½ MS Agar plates	2.15 g/l MS salts
	0.8% Phyto-agar (w/v)
	Adjusted to pH 5.7 with KOH

Treatments have been added to liquid media (55°) with the indicated concentration.

½ MS liquid medium	2.15 g/l MS salts
	Adjusted to pH 5.7 with KOH

Treatments have been added with the indicated concentrations.

Buffers and solutions

Protein extraction buffer	100 mM MOPS, pH 7.6
	100 mM NaCl
	40 mM ß-Mercaptorethanol
	10% SDS
	10% Glycerol
	4 mM EDTA
	2 mM AEBSF
	Protease inhibitor cocktail
Laemmli running buffer, 10x	50 mM Tris

	1.92 M Glycine
	1 % SDS
PAGE 4x resolving gels (10%)	9.9 ml H2O
	8.3 ml Acrylamide mix (30%)
	1.5 M Tris (pH 8.8)
	0.25 ml 10 % SDS
	0.25 ml APS
	0.02 ml TEMED
PAGE 4x stacking gels	6.8 ml H2O
	1.7 ml Acrylamide mix (30%)
	1.0 M Tris (pH 6.8)
	0.1 ml 10 % SDS
	0.1 ml APS
	0.01 ml TEMED
Blotting buffer	25 mM Tris
	192 mM Glycine
	0.1 % SDS
	20 % Methanol
Wash buffer	25 mM Tris
	0.1 % Tween 20
Amidoblack staining solution	10 % Acetic acid
	90 % Ethanol
	0.005 % Amidoblack
Polysome extraction buffer	0.2 mM Tris-HCl
	0.2 mM KCl
	25 mM EGTA

	35 mM MgCl2
	1% DOC
	1% PTE
	1% Brij-35
	1% Triton-X100
	1% NP-40
	5 mM DTT
	10 MG-132
	50 μg/ml cycloheximide
	50 μg/ml chloramphenicol
	1% EDTA-free protease inhibitors
Bieleski buffer	60% Methanol
	10% HCOOH
	30% H2O
GUS staining solution	0.2% Triton X-100
	50 mM NaPO4
	2 mM Potassium-Ferrocyanide
	2 mM Potassium-Ferricyanide
	2 mM X-Gluc

Instruments

Confocal microscope Nikon A1	Nikon Instruments; Tokyo, Japan
Microscope Axio Imager.M1	Carl Zeiss; Oberkochen, Germany
Milli-Q water system	Merck Millipore; Billerica, USA
Nanodrop ND-1000	Nanodrop; Wilmingron, USA
Pipetus Akku	Hirschmann; Eberstadt, Germany
Precision balance	Kern & Sohn; Balingen, Germany
Centrifuges	
Eppendorf 5430R	Eppendorf; Hamburg, Germany
Eppendorf 5424	Eppendorf; Hamburg, Germany

Tissue Lyser II	Quiagen; Hilden, Germany
Vortex-Genie 2	Scientific Industries; Bohemia, USA
Poly Klima (PK 520-LED)	polyklima; Freising, Germany
ECL-Imager	INTAS; Göttingen, Germany
qTOWER3	Analytic Jena; Jena, Germany
Multiple Gel Caster	Cytiva; Amersham, UK
SE250 Mighty Small II	Hoefer Inc.; Holliston, USA

Software

Office 365	Microsoft; Albuquerque, USA
R	https://cran.r-project.org/
R Studio	https://www.rstudio.com/
Image J/ Fiji	https://www.rstudio.com/
Inkscape	https://inkscape.org/de/
Package „drc“	(Ritz C, Strebig JC, 2016)
RNA STAR (v.2.6)	(Dobin et al., 2013)
StringTie (v.1.3.3)	(Pertea et al., 2015)
DESeq2(v.1.18.1)	(Love et al., 2014)
Fast QC	https://www.babraham.ac.uk/
Zotero	https://www.zotero.org/

Methods

Most experiments have already been described in (Janocha et al., 2021) and have been copied and modified from there.

Plant growth

Seeds were sterilized with 70% ethanol and 0.1% Triton for 10 min and afterwards washed twice with autoclaved water. Seeds were plated on 100 µm nylon meshes on top of 0.5x MS, 0.8% Phytoagar in square petri dishes. After plating, seeds were imbibed for 3 days at 4°C in darkness and transferred to growth cabinets where they were kept under continuous light at 22°C and grown vertically for 4 days. Subsequently, seedlings were transferred with the nylon meshes to 0.5x MS plates supplemented with the indicated treatments for the indicated durations.

Liquid culture

About 30–40 seeds, that were imbibed as described above, were sown in 3 ml 0.5x MS in petri dishes of 35 mm diameter. Plants were kept in darkness for three days after the induction of germination by 6 hr light treatment. The medium of two day old etiolated seedlings was supplemented with the indicated treatments. All stock solutions were 1000x concentrated and diluted in DMSO, therefore control plants were mock treated with the same volume of DMSO.

RNAseq

Seedlings were grown as described under growth conditions. 4 days after germination seedlings were transferred on a mesh to 0.5xMS plates containing either 2 µM AZD8055, 10 µM TORIN1, 20 µM KU63794 or equal volumes of DMSO as mock control. After 8 h, 30 mg of shoot tissue were harvested for each replicate and frozen in liquid nitrogen. 3 independent replicates were harvested for each condition. Total RNA was extracted with the Plant RNA Purification Reagent according to the instructions of the manufacturer, digested with TURBO DNAse and purified with RNeasy Mini Kit. Libraries were poly-(A) selected and analyzed with NEXTseq 500. For differential gene expression analysis reads were aligned with RNA STAR (v2.6) alignment tool with TAIR10 genome model as reference. Transcripts were assembled and counted with StringTie (v1.3.3) and statistical analysis was performed using DESeq2 (v1.18.1). GO term analysis was performed using PANTHER GO-Slim biologocical process overrepresentation test (Release 20210224) with Fisher test and bonferroni correction.

RNAseq raw data and count files are made available as *GSE197099* entry at GEO expression omnibus online repository.

Histochemical GUS staining

Four day old seedlings were harvested in 90% acetone and incubated at −20°C for at least 1 hr. Seedlings were washed with PBS and incubated in substrate buffer at 22°C over night. After staining, the seedlings were incubated with 60% and subsequently in 95% ethanol to remove chlorophyll.

Microscopy and fluorescence quantification

All images were obtained using Zeiss Imager M1, the Plan-APOCHROMAT 20x/0.8 objective and YFP- and GFP-specific filter sets. Procedures for fluorescent reporter activities of the double reporter were performed as described in (Pfeiffer et al., 2016). Each measurement was normalized to the median (set to 100) of the mock for experiments in the light or to the 6-BA treated samples for experiments performed in the dark.

Shoot regeneration assay

The assay has been performed as described in (Pernisova et al., 2018).

Western blot

Approximately 30 mg of shoot tissue were harvested, frozen in liquid nitrogen and ground with a tissue lyzer and glass beads in 2 ml eppies. Proteins were extracted with 1:4 ratio (mg/μl) adjusted to the exact fresh weight with 95°C hot denaturing buffer protein extraction buffer and boiled at 95°C for 5 min. Cellular debris was removed by two centrifugation steps (10 min, 14,000 rpm, RT). Equal volumes of the obtained extract were separated on a 10% SDS-PAGE gel and blotted to a PVDF membrane. Membranes were probed with anit-S6K1(phospho T449) polyclonal antibody to detect S6K1 phosphorylation (1:5000). S6K1/2 antibody was used to detect total S6K1 and S6K2 (1:5000). c-Myc antibody was used to detect c-Myc tagged CKX1 (1:1000). Anti-GFP antibody was used to detect GFP-ATG8 (1:5000). Membranes were blocked with BSA 5% or milk 5% according to the manufacturer instructions for 1 h while rigorously shaking. Species specifc secondary antibodies coupled with HRP were used at 1:20000 dilution. Between all steps membranes were washed with wash buffer three times 5 min each.

RT-qPCR

Total RNA was extracted as described under RNAseq. RNA integrity was confirmed on an agarose gel and the concentrations were determined with a nanodrop device. Equal amounts of RNA were used for oligo dT primed cDNA synthesis with the RevertAid First Strand cDNA Synthesis Kit. The qPCR reaction was set up using the SG qPCR Master Mix and run on a qTOWER3 PCR System with technical duplicates each.

Cytokinin response assay

Seedlings were grown as described under growth conditions. After 8 h of AZD8055 or mock treatment seedlings were sprayed with an atomizer with either 100 nM of trans-zeatin or 100 nM of isopentyladenine solution (0.015% Silwet L-77). After 30 min three independent replicates of shoots and roots were harvested separately for total RNA extraction and RT-qPCR analysis as described above.

Cytokinin profiling – LC-MS

For cytokinin profiling seedlings were grown as described for RNAseq analysis and 5 biological replicates of shoot tissue were harvested for each condition. The CK content was determined by ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (Svačinová et al., 2012), including modifications described by (Antoniadi et al., 2015). Briefly, samples (20 mg FW) were homogenized and extracted in 1 ml of modified Bieleski buffertogether with a cocktail of stable isotope-labeled internal standards (0.25 pmol of CK bases, ribosides, N-glucosides, and 0.5 pmol of CK O-glucosides, nucleotides per sample added). The extracts were purified onto an Oasis MCX column (30 mg/1 ml, Waters) and then analyzed using using an Acquity I-class system (Waters, Milford, MA, USA) combined with a mass spectrometer Xevo™ TQ-XS (Waters, Manchester, UK). Data were processed with Target Lynx V4.2 software and final concentration levels of phytohormones were calculated using isotope dilution method (Novák et al., 2008).

Physiology

Seeds were singled out on 0.5xMS, 0.9% Phytoagar plates and imbibed for three days at 4°C in the dark. Plates were kept horizontally in long day conditions at 22°C for four days. ~ 40 single seedlings at the same developmental stage and of similar size were selected and transferred to plates containing the indicated AZD8055 concentrations and grown for seven more days before shoot fresh weight was measured. For the measurements, seedling shoots

were removed and weighed in batches of 5 – 10 shoots. Afterwards the average weight was calculated for each batch.

Statistical testing

Statistical analysis for experiments shown in Fig. 9a+e, linear mixed models were ("lme4" package "R") were generated with Genotype (Freshweight ~ AZDconcentration*Genotype + (1|Experiment)) or without Genotype (Freshweight ~ AZDconcentration + (1|Experiment)) and compared with ANOVA to calculate the p-value for significant interaction. For Fig. 9c, a linear model with (Freshweight ~ AZDconcentration*Treatment) was compared with (Freshweight ~ AZDconcentration) using ANOVA. Datasets were previously evaluated for extreme outliers, normality assumptions and heteroscedasticity. Pairwise t-tests have been performed for group comparisons. Statistical analysis was performed in R (v4.0.2) with unnormalized data. ED50 values were calculated using the "drc" package in R (Ritz C, Strebig JC, 2016).

Data that was not normally distributed was tested with Wilcoxon rank test and Hochberg correction in R. Normally distributed data was tested for heteroscedasticity and two-tailed students t-test with equal or unequal variance have been performed accordingly.

CHX chase assay

P35S:cMyc-CKX1 seedlings were grown as described under growth conditions. 8 h after transfer to 2 µM AZD8055 the plates were flooded with 50 ml 200 µM cycloheximide and 2µM AZD8055 solution (0.015% Silwet L-77) for 0, 2, 4 and 8 h and shoots were harvested for western blot analysis as described above.

Polysome fractionation

200 mg plant material grown as described under growth conditions was homogenized by rotating at 4°C in 600 ul polysome extraction. Extracts were centrifuged at 16000xg at 4°C for 10 min. 300 µl supernatant was loaded to 7-47% sucrose gradient and centrifuged at 38000x rpm for 3 hours in a Beckmann SW41Ti rotor. The gradient was fractionated after recording the absorbance at 254 nm. RNA was precipitated from 1 ml fraction by mix and incubation with one volume of 8 M guanidine-HCL and two volumes of absolute ethanol at -20°C over night followed by centrifuge at max. speed for 1 hour. RNA pellet was resuspended with 50 µl DEPC water. 100 ng RNA was used for cDNA synthesis (SuperScript IV reverse transcriptase (ThermoFisher, 18090050) which was subsequently analysed by qRT-PCR as described above.

Grafting

Grafts were generated as described in (Melnyk, 2017). 7 days post grafting seedlings were transferred to soil and long day conditions.

GFP-ATG8 cleavage assay

P35S:GFP-ATG8 seedlings were grown as described under plant growth and 4 DAG were transferred to 2 μM AZD8055 or mock medium for 8 h. Shoots and roots were harvested separately and processed as described under western blot.

Results

Transcriptome analysis

After the TOR network has been identified as an important upstream regulator of WUS (Pfeiffer et al., 2016), I wanted to understand how changes of TOR kinase activity are conveyed to control WUS expression. As *tor null* alleles display embryonic lethality and plants seem to be generally insensitive to TOR inhibition with rapamycin (Xiong & Sheen, 2012), I decided to base my analysis on the effects of second-generation active site inhibitors of the TOR kinase (asTORis) (Montané & Menand, 2019). To identify candidate pathways that could serve as downstream effectors of TOR, I wanted to utilize transcriptome analysis of TOR inhibited seedlings. The only available dataset for asTORis at this time was lacking biological replicates and hence bared high uncertainty (Dong et al., 2015). Moreover, the treatment duration in this study was 24 h, which made it likely that many differentially expressed genes (DEGs) were not primary target genes. In addition, I wanted to identify genes that mediate shoot development and other transcriptomes were derived from whole seedlings, potentially disguising or diluting interesting DEGs that are antagonistically regulated in shoots versus roots. Moreover, no data about the specificity of the different asTORis was available and I reasoned that I could enrich my data for high confidence TOR targets by combining transcriptomes of three different asTORis. I chose AZD8055, TORIN1 and KU63794 as they have been demonstrated to cause substantial inhibition of shoot development at 2 μM, 10 μM and 20 μM respectively (Dong et al., 2015). I monitored the effect of the respective inhibitors on phosphorylation levels of S6K1 and found that 8 h after transfer of 4-day old seedling shoots grown under continuous light, phosphorylation of S6K1 dropped substantially compared to mock (Fig. 1a). In contrast, 6 h after transfer S6K phosphorylation was increased for all three inhibitors, indicating that TOR activity dropped between 6 h and 8 h. Hence, I decided to analyze the transcriptomic changes of the three inhibitors 8 h after transfer using NEXTseq.

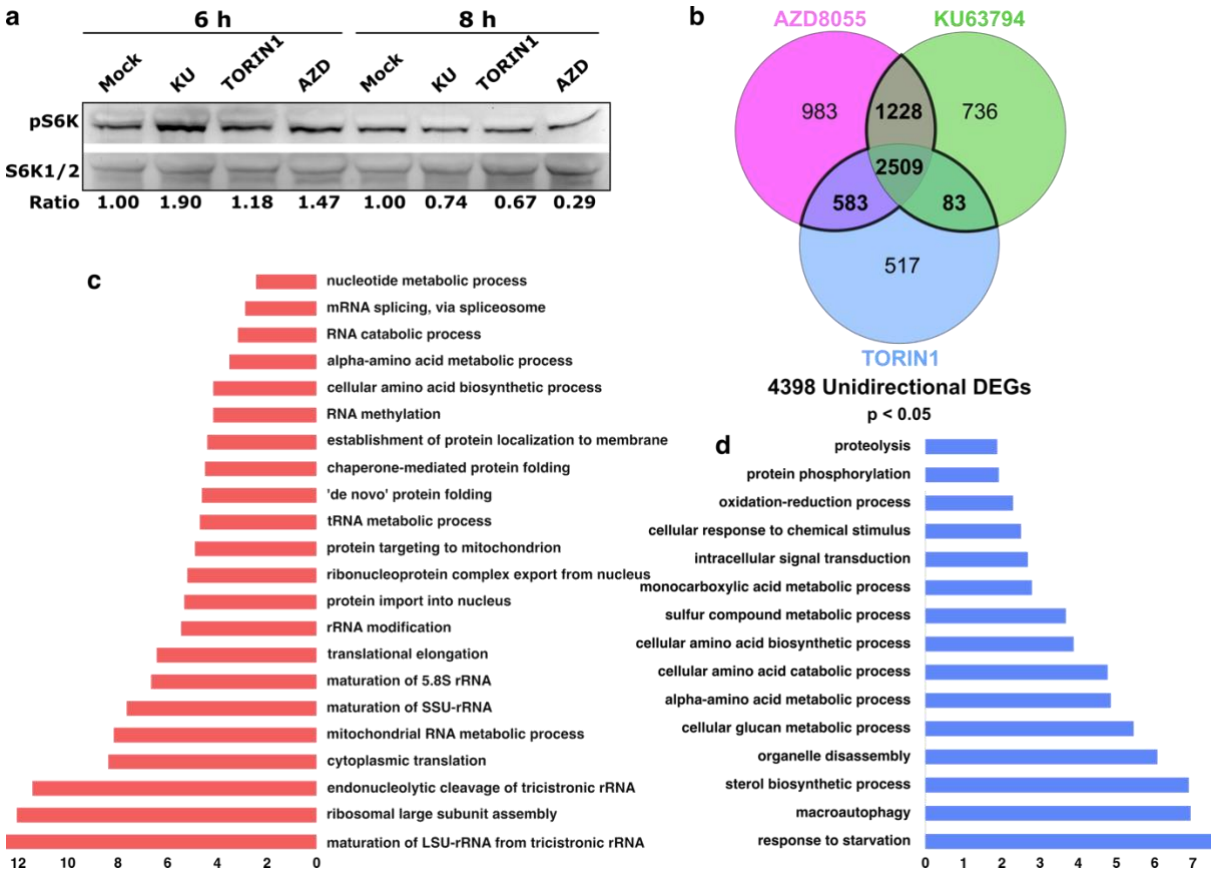


Figure 1 Shoot specific TOR transcriptome: a) Western blot of Arabidopsis shoot tissue extracts from 4-day old seedlings treated with different TOR inhibitors for the indicated time. Membranes were probed with serum detecting TOR specific phosphor-epitope (T449) or total protein of S6K1/2. 2 µM AZD8055, 10 µM TORIN1, 20 µM KU63794. Ratios were calculated between pS6K (No.9205, Cell Signaling) and S6K total band intensities and normalized to the respective Mock sample which was set to 1. **b)** Venn diagram of differentially expressed genes obtained from RNAseq of shoot tissue from 4 day old seedlings treated with different TOR inhibitors for 8 h. **c)+d)** Overrepresented GO terms among c) downregulated and d) upregulated DEGs from b). Scale indicates fold enrichment relative to the Arabidopsis genome (TAIR10).

I utilized the DESeq2 pipeline (Love et al., 2014) to identify DEGs between the inhibitor and the mock treated control samples and found 6639 genes differentially regulated across all samples. 3266 (49.2%) of the DEGs were downregulated and 3373 (50.8%) were upregulated compared with the mock (Fig. 1b and Table S1). AZD8055 treatment changed the expression of 5303 genes, KU63794 of 4556 genes and TORIN1 of 3692 genes which is in line with the stronger effect of AZD8055 on S6K1 phosphorylation (Fig. 1a). Comparison of the different transcriptomes revealed that 2509 (37.7%) of the genes were commonly regulated by all three inhibitors and 4403 (66.3%) were regulated by at least two inhibitors. The differences between the three substances can be explained by their unique selectivity- and efficacy profiles (Montané & Menand, 2019). I restricted my further analysis to genes that were differentially regulated by at least two of the three inhibitors and were regulated in the same direction and

thereby obtained a list of 4398 high confident TOR target genes (Fig. 1b + Table S1). Gene ontology (GO) enrichment analysis of the up- and down- regulated genes revealed many functional categories already associated with TOR function (Fig. 1c + d). Among the downregulated genes particularly categories associated with regulation of translation, ribosomal RNA and tRNA processing but also mRNA splicing, amino acid- and nucleotide metabolism were enriched (Dobrenel et al., 2016; Shi et al., 2018). Interestingly, also categories related to protein folding were enriched, a process which so far has not been linked to TOR function, together with RNA methylation which has only recently been described in mammals as being controlled by TOR (Cho et al., 2021; Tang et al., 2021). Response to starvation was the most enriched category among the upregulated genes (Fig. 1d), together with categories related to catabolism such as macro-autophagy, organelle disassembly or proteolysis which are well characterized functions of TOR (Pu et al., 2017). Also, most of the other enriched categories related to lipid- starch-, carbon-, sulfur-, and amino acid metabolism have already been linked to TOR function (Caldana et al., 2013; Dobrenel et al., 2016; Dong et al., 2017) suggesting that I have obtained a valid dataset.

I also compared my gene list with TOR target genes generated by other groups and saw a significant overlap with studies analyzing the transcriptomes of AZD8055 or TORIN2 treated whole seedlings (Dong et al., 2015; Scarpin et al., 2020). After adjusting for the same expression cut off, 63 % of TORIN2 regulated genes were also differentially regulated in my dataset even though the experimental setup differed quite substantially from mine (Fig. 2b). The overlap with the AZD8055 treated whole seedlings from (Dong et al., 2015) was lower (Fig. 2a), as only around 43.2% of my genes were contained in their list. The TORIN2 dataset was generated after 2 h of treatment, whereas the AZD8055 dataset was generated after 24 h of treatment and both have treated whole seedlings. The larger overlap with the TORIN2 dataset suggests that the treatment duration might have a larger influence on the transcriptome than the particular inhibitor and indicates that changes in TOR activity initiate multi-layered transcriptional cascades. This is corroborated by the comparison of my dataset with RNAi data of inducible amiTOR lines (Fig. 2c) (Caldana et al., 2013). There, 3 or 6 days after induction of the amiTOR construct only 33 or 91 genes respectively were commonly regulated when compared with my TOR target genes. Noteworthy, the amiTOR dataset relied on microarrays and targeting TOR mRNA is mechanistically different from kinase inhibition, as this might result in altered stoichiometries of a megadalton protein complex. In sum, the overlap of my dataset with previously published data and the high agreement of the enriched GO categories

with already known TOR functions is a good indication that I have obtained a list with high confidence TOR target genes.

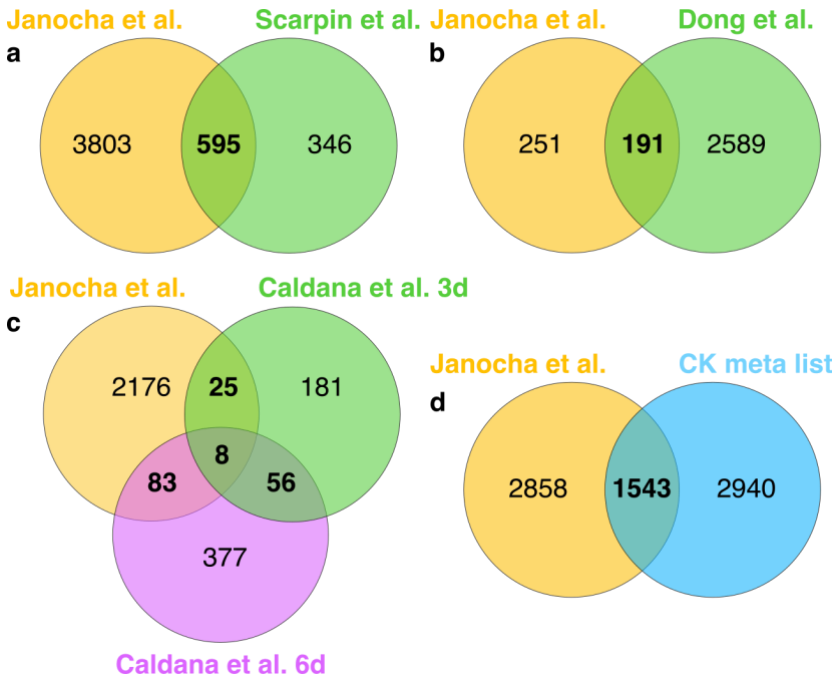


Figure 2: Comparative analysis oft the TOR shoot transcriptome: a) Venn diagram of shoot specific DEGs from this study compared with TORIN2 regulated DEGs from (Scarpin et al., 2020). Hypergeometric test reveals 4.5 fold enrichment of the overlap with p = 2.6 e-276. **b)** Venn diagram of shoot specific DEGs from this study with compared with AZD8055 regulated DEGs from (Dong et al., 2015). Hypergeometric test reveals 4.86 fold enrichment of the overlap with p = 8.86 e-84. Expression cutoff is log2 fold change > 1. **c)** Venn diagram of shoot specific DEGs from this study compared with DEGs of inducible tor RNAi lines from (Caldana et al., 2013) after 3d of induction (green) or 6d of induction (purple). Hypergeometric test reveals 1.22 fold enrichment which is not significant (p = 0.3) after 3d and significant (p = 1.07 e-07) 1.74 fold enrichment after 6d of tor RNAi induction. Expression cutoff is log2 fold change > 0.5. **d)** Venn diagram of shoot specific DEGs from this study compared with a meta list of genes frequently de-regulated by CK obtained from (Bhargava et al., 2013).

The root transcriptome

I also analyzed the transcriptome of the respective roots from my mock and AZD8055 treated seedlings and found 3337 genes differentially regulated (Fig. 3 + Table S2). Among the upregulated genes, again response to starvation and categories related to catabolism were most enriched together with glutathione metabolism, being closely related to sulfur- and glucosinolate metabolism which both have been reported in relation to TOR (Fig. 3b) (Dong et al., 2017; Malinovsky et al., 2017). Interestingly, among the downregulated genes photosynthesis was the most enriched GO category together with the related categories pigment biosynthesis and response to light (Fig. 3c). This was rather unexpected for a root transcriptome which is not a photosynthetic tissue. This result could be an artifact of root

illumination which has been reported in many other circumstances (Cabrera et al., 2021). Noteworthy, TOR was found to be involved in regulation of photosynthesis in a previous study where the transcriptome has been generated with whole seedlings (Dong et al., 2015). However, I also found the expected TOR related categories such as ribosome biogenesis, translation and rRNA processing to be repressed (Fig. 3c). Consequently, I found 1335 genes being commonly regulated between roots and shoots (Fig. 3a). Intriguingly, in roots ER unfolded protein response (UPR) and protein folding were among the enriched GO categories and I found many UPR marker genes repressed, like CALNEXIN1 (CNX1), CALRETICULIN (CRTs), BIP1+2 together with many heat-shock protein and other chaperones (Table S2). As the UPR is usually upregulated during folding stress in the ER and TOR inhibition reduces translation, this might result in a reduced folding burden in the ER and hence less of the UPR machinery would be required. However, in the shoot transcriptome I found many UPR marker genes to accumulate (Table S1), pointing towards an inverse regulation of the UPR in shoots versus roots. Recent studies confirm that TOR is involved in ER and UPR regulation (Angelos & Brandizzi, 2021; Cao et al., 2019).

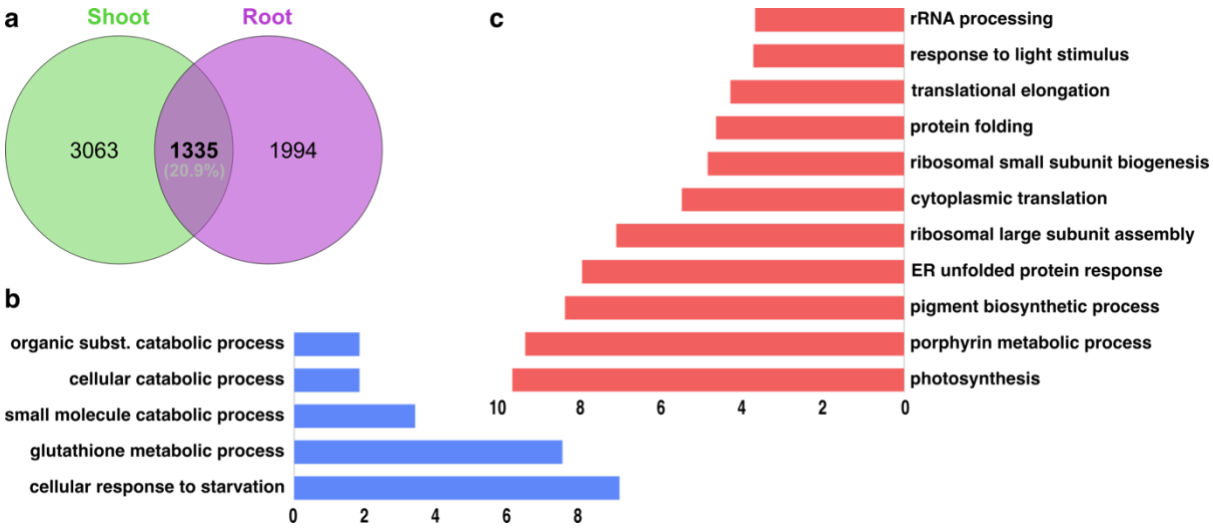


Figure 3: The TOR root transcriptome: a) Venn diagram of differentially expressed genes obtained from RNAseq of root tissue from 4 day old seedlings treated with different AZD8055 for 8 h compared with the DEGs obtained from the shoot transcriptome analysis. The roots used for this analysis correspond to the AZD8055 shoots from Fig.1. b)+c) Overrepresented GO terms among c) downregulated and b) upregulated DEGs from the root transcriptome of a). Scale indicates fold enrichment relative to the Arabidopsis genome (TAIR10).

Cytokinin signaling is deregulated after TOR inhibition

More detailed inspection of my shoot dataset revealed many DEGs related to hormone signaling pathways (Fig. 4a). Genes related to ABA (~180 genes) and Auxin (~100 genes) were most prevalent, but also genes related to cytokinin (CK) (~80 genes), jasmonic acid (JA) (~75

genes), ethylene (ET) (~70 genes), gibberellic acid GA (~40 genes) and brassinosteroids (BR) (~20 genes) were deregulated. It appeared that CK and JA related DEGs were mostly downregulated while for the DEGs related to other pathways DEGs were equally up- or downregulated. This was particularly interesting, as CK signaling is known as important instructive signal for the SAM and has been previously characterized as major determinant controlling SAM size, stem cell proliferation and *WUS* expression (Buechel et al., 2010; Landrein et al., 2018; Osugi et al., 2017). I mapped the CK related DEGs according to their characterized functions in CK signaling and found pathway wide deregulation (Fig. 4b). In roots as well as in shoots genes related to CK biosynthesis (*IPT1,-9, CYP735A2, LOG1,-2,-3,-5,-7,-8*), CK translocation (*PUP1,-14,-16, ENT6, ABCG14*), CK signal perception and -transmission (*AHK2,-3,-4, AHP1,-2,-3*) but also transcriptional regulators (*type-B ARR1,-2,-10,-14*) and response genes (*type-A ARR3,-4,-5,-6,-7,-9, CRF6,-10,-11*) were deregulated after TOR inhibitor treatment. Evidently, the effect of TOR inhibition had very different effects on CK signaling in roots and shoots, which is in line with the divergent functions of CK in both tissues. CYP735A2 is the enzyme responsible for the conversion from *i*PRMP precursors to *tZ*RMP precursors and was strongly repressed in shoot and root samples pointing towards globally reduced tZ biosynthesis. At the same time expression of IPT1, LOG5 and LOG8 was increased suggesting enhanced synthesis of *i*PRMP and conversion of monophosphate precursors to their active base forms. IPT9 expression was repressed in shoots and roots pointing towards reduced isopentylation of tRNAs. For the root LOGs, the isoforms *LOG1*, *-2* and *-3* were downregulated and *LOG7* and *-8* were upregulated. Even though the observed changes in biosynthetic pathway genes suggest changed CK homeostasis, the mere transcriptome profiles do not allow conclusions about the actual metabolite levels. However, my results suggest changes in the metabolic flux through the CK biosynthesis pathway in roots as well in shoots. In line with this, expression of several CK transporters was elevated in shoots, particularly the CK importer *PUP14* which has been recently characterized as a negative regulator of CK signaling (Zürcher et al., 2016). Interestingly, *ABCG14* a CK exporter responsible for root to shoot translocation of CKs was inversely regulated in shoot versus root samples, indicating that CK transport from roots to shoots might be affected as well (Ko et al., 2014). Also, CK receptors were differentially expressed when TOR was inhibited. In shoots only *AHK4* expression appeared slightly repressed while in roots all three *AHKs* showed elevated expression levels. Similarly, expression of three *AHPs* was elevated in roots while in shoots *AHP1* was down- and *AHP2* was upregulated. Expression of several *type-B ARRs* was elevated in roots as well as in shoots. In roots the CK signal transmission machinery appeared

consistently upregulated suggesting increased sensitivity towards CK while no consistent pattern was observed in shoots. However, as CK signaling output is commonly evaluated by *type-A ARR* expression and strikingly, all 6 differentially expressed members of this gene family were repressed in shoots, this clearly indicated reduced CK signaling output in shoots. In sum, my transcriptome data suggested that CK signaling was reduced in shoots but did not allow definite conclusions about CK signaling in roots.

I sought to clarify the effects of TOR inhibition on CK signaling utilizing the synthetic TCS promoter element commonly used as a readout for CK pathway activation (Zürcher et al., 2013). The pTCSn:GUS reporter line showed strong GUS signal in the SAM which was strongly reduced following TOR inhibition with AZD8055 (Fig. 4c). In contrast, pTCSn:GUS expression appeared strongly elevated in the whole root vasculature. This confirmed the predictions made with the transcriptome data, whereafter the net effect of TOR inhibition is a strong reduction of CK signaling in shoots, while the signaling is enhanced in roots. This is consistent with the growth promoting function of CK in shoots versus the growth repressive function CK in roots and is in line with the notion of TOR as a promoter of growth.

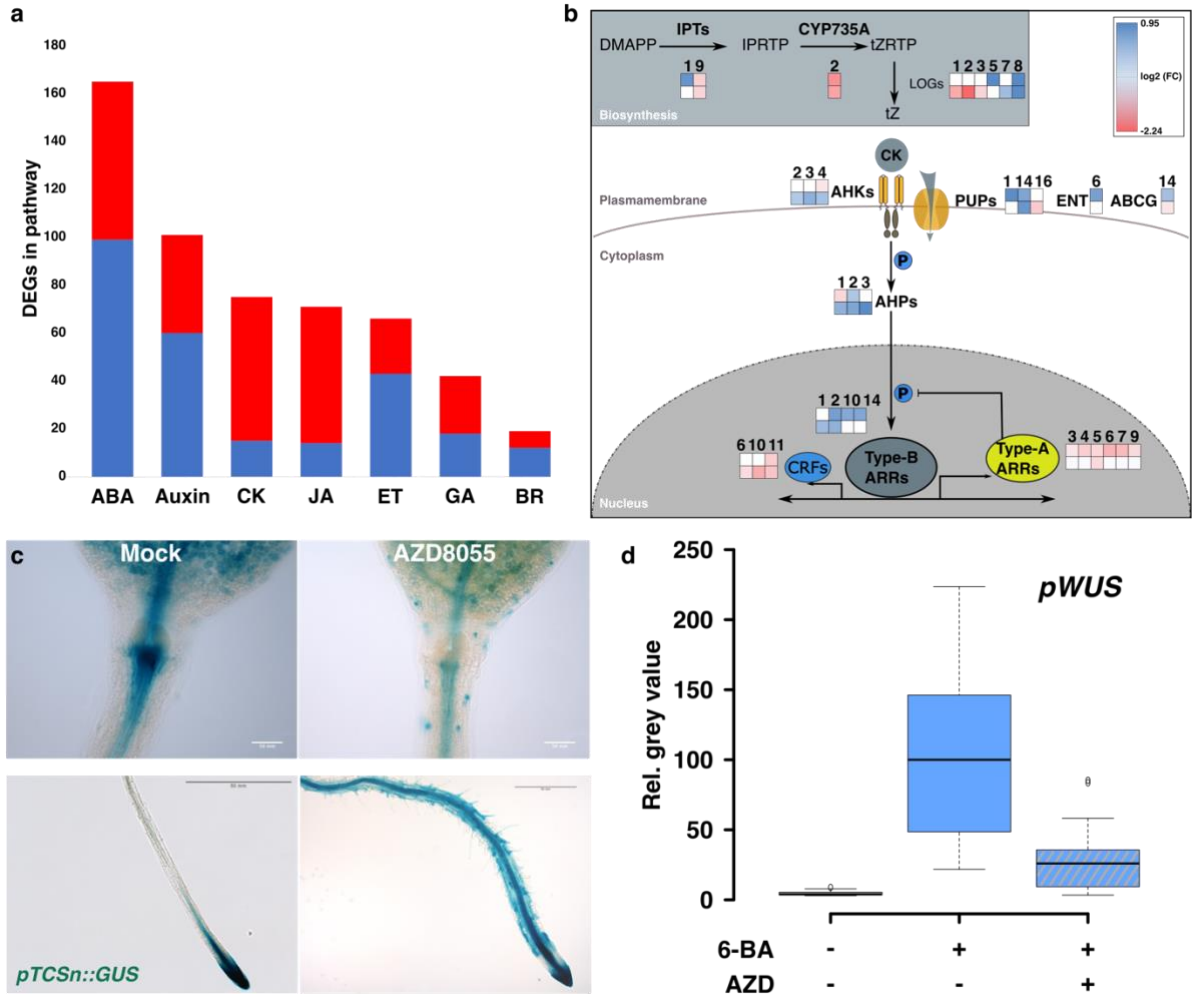


Figure 4: TOR inhibition interferes with CK signaling output: a) Gene count of DEGs from RNAseq analysis annotated with hormone signaling function from GO term analysis. ABA = abscisic acid, JA = jasmonic acid, GA = gibberellic acid, BR = brassinosteroids. **b)** Schematic representation of the CK signaling pathway. Color code represents log fold change value obtained from RNAseq analysis. The numbers over the boxes indicate the isoform number of the respective gene. Upper lane of color panels indicates log2(FC) in shoots and lower panels in roots. IPT = isopentyltransferase, LOG = lonely guy, AHK = Arabidopsis histidine kinase, AHP = Arabidopsis histidine phosphotransferase, ARR = Arabidopsis response regulator, PUP = purine permease. **c)** Representative microscopic image of *pTCSn:GUS* reporter line treated with either DMSO or AZD8055 for 24 h. **d)** Quantification of *pWUS:3xVenus:NLS* reporter signal from 5 day old etiolated seedlings treated with 0.5 µM 6-benzyladenine and 2 µM AZD8055 for 3 days. Significant differences between Mock-BA(p=4.05e-13), Mock-BA+AZD (p=5.88e-08) and BA-BA+AZD (p=1.42e-07) were calculated with Wilcoxon rank sum test with Hochberg correction. n=22-27.

While my results confirmed altered CK signaling, I wanted to investigate if also the functional output of CK signaling was affected and hence monitored the effect of AZD8055 treatment on the outcome of several functional CK assays. I was particularly interested if TOR inhibition altered the ability of CK to induce *WUS* expression and therefore utilized a pWUS:3xVenus-NLS/pCLV3:mCherry-NLS double-reporter line (Pfeiffer et al., 2016). As shown before 6-BA treatment strongly induced *WUS* expression in etiolated seedlings (Fig. 4d) (Pfeiffer et al.,

2016). This effect was almost completely abolished when supplemented together with AZD8055, indicating that TOR inhibition interferes with the ability of CK to induce *WUS*. The same repressive effect of AZD8055 treatment was observed for CK induced *type-A ARR* expression in etiolated seedlings (Fig. 6a). There, induced expression of ARR5, -6, -7 and -15 was reversed when 6-BA was applied together with AZD8055, confirming a negative effect on the expression of several CK target genes.

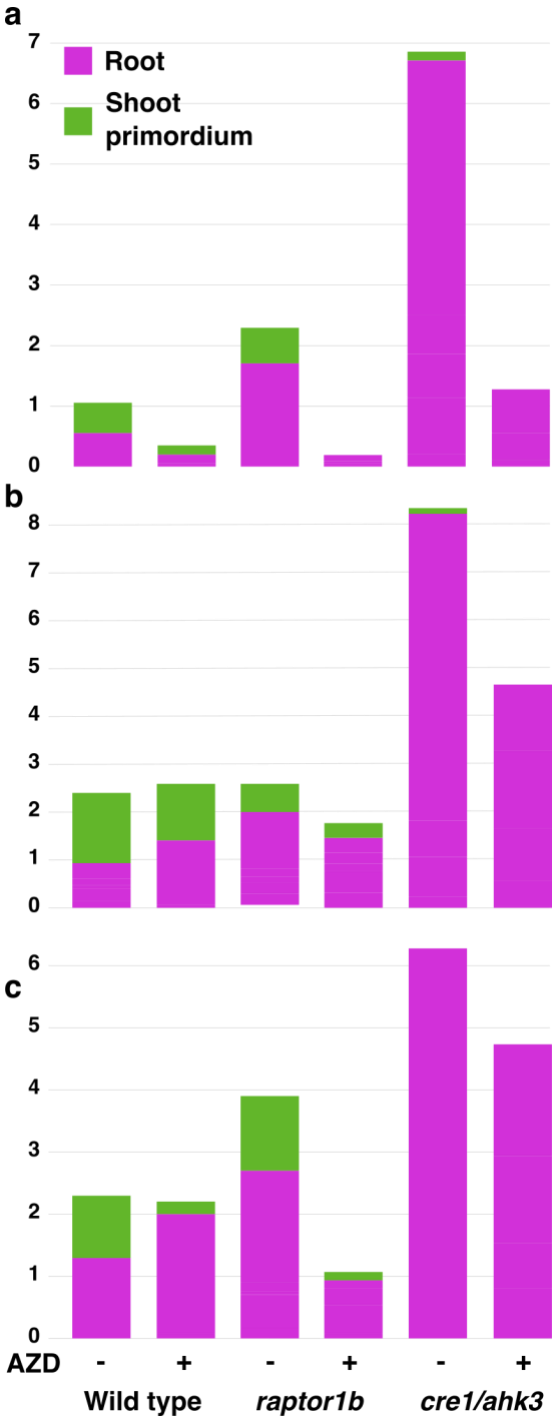


Figure 5: TOR inhibition affects CK mediated acquisition of shoot identity: Counts of different root and shoot primordia from shoot regeneration assays. Etiolated hypocotyls were excised and transferred to shoot induction media (SIM) containing auxin and different cytokinins. Quantification was performed 7 days after transplantation to SIM only or to SIM supplemented with 2 µM AZD8055. **a)** 100ng/ml naphthalene-acetic acidNAA + 300ng/ml Kinetin **b)** 100ng/ml NAA + 300ng/ml isopentlyadenine **c)** 100ng/ml NAA + 300ng/ml trans-Zeatin.

CK is frequently used in shoot regeneration assays, where hypocotyl explants of etiolated seedlings are transferred to shoot induction medium (SIM) supplemented with auxin and cytokinin (Pernisova et al., 2018). In this setup, auxin induces the emergence of root primordia from internal tissues, a process which is inhibited by cytokinin. Subsequently, cytokinin reprograms the emerging root primordia to acquire shoot fate and the number and ratios of root- and shoot primordia allow to infer functional changes in auxin and CK signaling. To this end, I transferred hypocotyl explants from etiolated wild type, *raptor1b* or *cre1/ahk3* seedlings to SIM, containing naphtalene-acetic acid (NAA) together with different CK derivatives and AZD8055 (Fig. 5a - c). Compared with wild type hypocotyls, the *cre1/ahk3* CK receptor double mutant hypocotyls produced up to seven-fold more root primordia and almost no shoot primordia, confirming the repressive function of CK signaling on root primordia emergence and the positive role of CK for the acquisition of shoot fate. Depending on the CK derivative, *raptor1b* mutant hypocotyls produced more primordia and had a lower ratio of shoot primordia compared with the wild type, both pointing towards impaired CK signaling. Interestingly, AZD8055 treatment had different effects compared with the *raptor1b* mutation, as the number of induced root primordia was either unchanged or even reduced compared with the respective control, indicating that auxin signaling might be additionally impaired. However, the ratio of shoot- to root primordia produced also decreased following TOR inhibition pointing towards reduced CK signaling output in response to AZD8055. In sum, this assay confirmed the negative effect of reduced TOR activity by AZD8055 treatment or by abolishing its co-factor RAPTOR1B on CK functional output but also shows that both approaches to reduce TOR activity led to slightly changed outcomes. Moreover, the effect of TOR inhibition on shoot regeneration seemed to depend on the respective CK species as the obtained results slightly differed between Kinetin, *t*Z and *i*P.

TOR interferes upstream of type-B ARR transcription factors

After having confirmed that reduced TOR activity negatively affected CK function in shoots, I wanted to narrow down at which stage in the CK signaling pathway TOR interfered with. To test whether the functionality of type-B ARR transcription factors was impaired by TOR inhibition, I generated a cross between the pWUS:3xVenus-NLS reporter line and a line expressing a truncated dominant version of ARR1 coupled to a glucocorticoid receptor (ARR1∂DDK:GR) that enables controlled nuclear translocation of the protein after dexamethasone (DEX) treatment (Sakai et al., 2001). Upon DEX treatment *WUS* reporter signal became visible in almost every cell of the seedling and persisted when DEX was applied together with AZD8055 (Fig. 6b). This confirmed that WUS is a direct target gene of type-B

ARRs and implied that TOR activity does not attenuate ARR1 activity. Consistently, also ARR1∂DDK:GR induced expression of type-A ARRs was not impaired when DEX was applied together with AZD8055 (Fig. 6c). Thus, I hypothesized that TOR activity likely interferes with CK signaling upstream of type-B ARRs transcriptional activation.

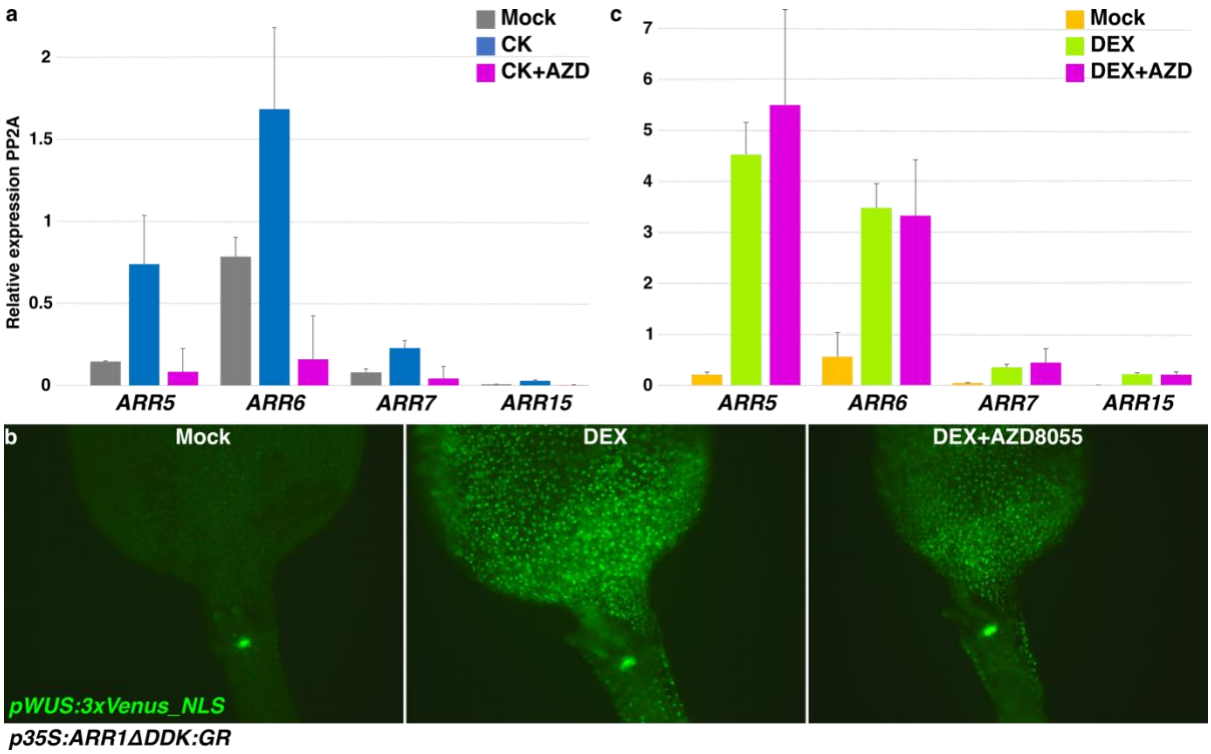


Figure 6: TOR does not impair type-B ARR function: a) q-RT-PCR of 3 day old etiolated seedling shoots grown for 3 days on mock, 0.5 µM 6-BA or 6-BA + 2 µM AZD8055 supplemented medium. Error bars represent standard deviation of 3 biological replicates. **b)** Representative microscopic images of *p35S:ARR1∂DDK:GR* crossed with *pWUS:3xVenusNLS*. 3 day old light grown seedlings were subjected to the indicated treatments for 24 h. 10 µM dexamethasone (DEX), 2 µM AZD8055. **c)** q-RT-PCR of 3 day old p35S:ARR1∂DDK:GR shoots grown for 1 day on either Mock, 10 µM dexamethasone (DEX) or DEX + 2 µM AZD8055. Error bars represent standard deviation of 3 biological replicates.

To address the possibility of reduced sensitivity towards CK through impaired receptor signaling or reduced signal transmission through AHPs, I monitored expression of ARR5 in response to the endogenous CK derivatives iP and tZ after pre-incubation of seedlings on AZD8055 for 8h. To resolve subtle differences in CK sensing it is important to not saturate the hormone response wherefore I chose to spray the preincubated seedlings with 100 nM solution of the respective CK and analyzed *ARR5* levels 30 min after treatment by q-RT-PCR. I utilized the *cre1/ahk3* CK receptor double mutant as a negative control. As expected, treatment with *tZ* induced a solid transcriptional response of *ARR5* compared with the negative control in roots as well as in shoots when seedlings were pre-incubated on mock

medium (Fig. 7a + b). In contrast, pre-incubation on AZD8055 supplemented medium reduced *ARR5* induction in shoots and fully repressed the transcriptional response in roots. In contrast, the root transcriptional response to *i*P remained unchanged even after pre-incubation on AZD8055. Interestingly, shoots did not respond to the *i*P treatment even on mock medium, which is in line with the reduced potential of *i*P to contribute to shoot development previously reported (Kiba et al., 2013). Since all CK receptors have similar or even higher affinities for *t*Z compared to *i*P, I concluded that CK sensing at the receptor level was unaltered after TOR inhibition (Romanov et al., 2006). Moreover, the reduced or completely abolished sensing towards exogenous *t*Z and the simultaneously unchanged response to *i*P led me to the conclusion that the exogenous *t*Z was either selectively degraded or sequestered.

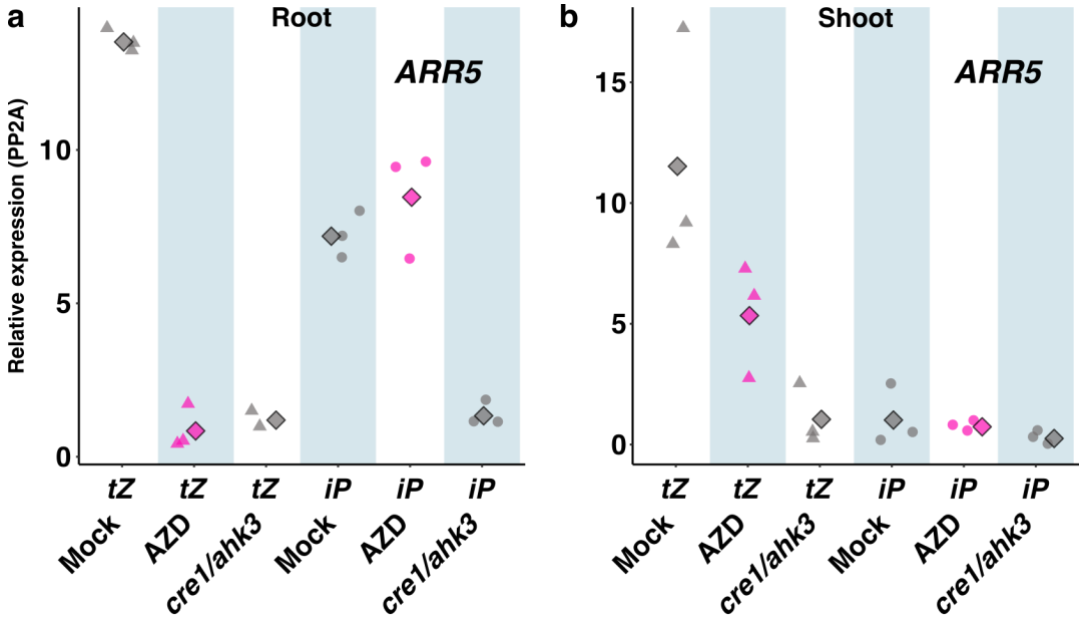


Figure 7: TOR controls CK homeostasis: a)+b) Relative expression values, normalized to PP2A obtained with q-RT-PCR from a) root and the respective b) shoot tissue are shown. 4 day old seedlings preincubated on AZD8055 or mock for 8h and subsequently sprayed with either 100 nM trans-zeatin (tZ) or 100 nM isopentyladenine (iP) solution for 30 min. Data points show expression values from biological replicates (triangles and circles) together with the calculated mean (rhombus).

Cytokinin metabolic profiling

My previous results pointed towards altered availability of specific CK derivatives after TOR inhibition. Together with the observation of several differentially expressed genes in CK biosynthesis (Fig. 4b), I was prompted to measure the levels of several CK metabolites. After extraction, CK metabolites were determined by LC-MS measurements. Strikingly, I observed

drastic effects of AZD8055 treatment on numerous CK metabolites (Fig.8a - d). The strongest effects occurred on *c*Z derivatives which all accumulated up to six-fold after TOR inhibition in shoots as well as in roots (Fig. 8b). cZ does not possess relevant activity in classical CK response assays which is consistent with the limited potential to induce expression of the *pWUS* reporter (Fig. 8d) (Gajdošová et al., 2011; Schäfer et al., 2015). However, as *c*Z species are thought to originate from isopentylated tRNAs this drastic increase pointed towards increased turnover of tRNAs (Kasahara et al., 2004). While TOR has been implicated in tRNA biosynthesis before (Shor et al., 2010), there are no reports about TOR controlling tRNA stability. The low abundant dihydrozeatin derivates DZR accumulated in shoots together with DZRMP in roots while the conjugate DZ7G was slightly depleted in shoots and roots (Fig. 8c). Since DZ is only present in minute amounts in plant tissues its relevance for CK signaling is assumed to be limited, particularly when considering its low affinities towards CK receptors (Romanov et al., 2006). *i*PRMP precursors were slightly depleted in roots and shoots just as *i*PR and *i*P were depleted in roots, whereas shoots accumulated two-fold levels of *i*P (Fig. 8d). I also observed a consistent reduction of several *t*Z derivatives in shoots as well as in roots (Fig. 8a). The strongest relative reduction occurred for *t*ZR and *t*ZRMP which were reduced four- and eight-fold respectively in shoots where also the active *t*Z bases were reduced two-fold. In several studies *t*Z has been shown to be the most important CK for shoot development and meristem size regulation (Kiba et al., 2013; Landrein et al., 2018; Osugi et al., 2017) and it was thus likely that such a drastic reduction in *t*Z levels would sufficiently explain the repressive effects of TOR inhibition on *WUS* expression and shoot growth.

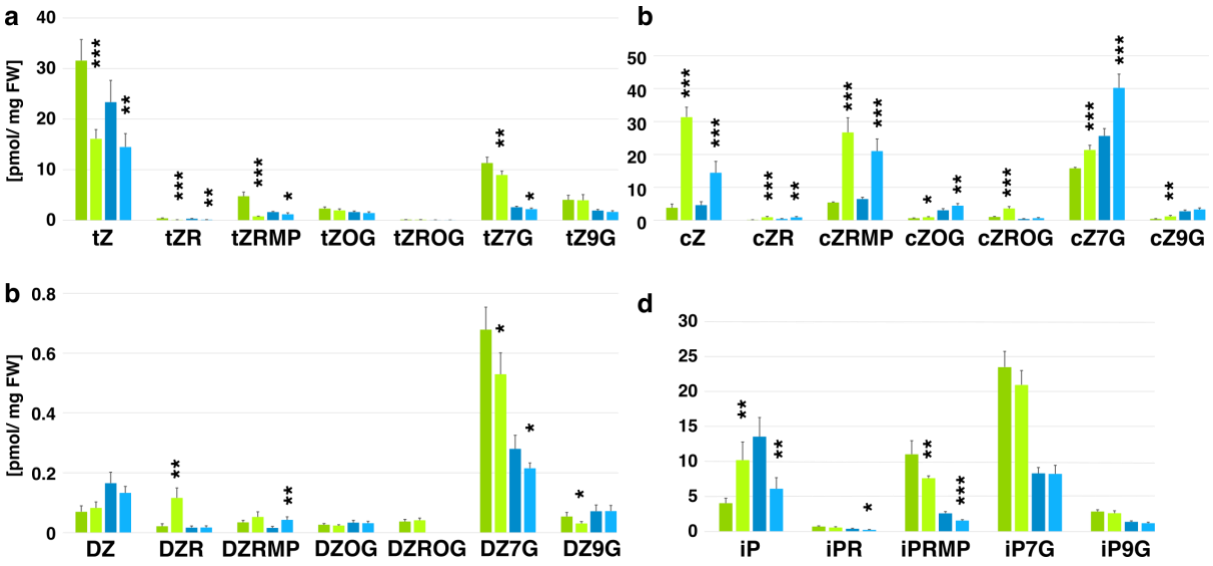


Figure 8: a)-d) Quantification of different CK metabolites measured by LC-MS in 4 day old seedlings treated with AZD8055 (shoot = light green; root = light blue) or mock (shoot = dark green; root = dark blue) for 8 h. Asterisks indicated significant differences of the AZD8055 treated sample

compared with the respective mock calculated with two-tailed t-test (*, **, and *** correspond to P-values of 0.05 > p > 0.01, 0.01 > p > 0.001, and p < 0.001, respectively). Error bars indicate standard deviation. n = 5. Data for this experiment was jointly produced with Ondrej Novak and Miroslav Strnad. Abbreviations: tZ = trans-zeatin, tZR = trans-zeatin riboside, tZRMP = trans-zeatin riboside-5´monophosphate, tZOG = trans-zeatin O-glucoside, tZROG = trans-zeatin riboside O-glucoside, tZ7G = trans-zeatin-7-glucoside, tZ9G = trans-zeatin-9-glucoside, iP = isopentyladenine, iPR = isopentyladenosine, iPRMP = isopentyladenosine-5´monophosphate, iP7G = isopentyladenine-7-glucoside, iP9G = isopentyladenine-9-glucoside DZ = dihydrozeatin, DZR = dihydrozeatin riboside, DZRMP = dihydrozeatin riboside-5´monophosphate, DZOG = dihydrozeatin O-glucoside, DZROG = dihydrozeatin riboside O-glucoside, DZ7G = dihydrozeatin-7-glucoside, DZ9G = dihydrozeatin-9-glucoside, cZ = cis-zeatin, cZR = cis-zeatin riboside, cZRMP = cis-zeatin riboside-5´monophosphate, cZOG = cis-zeatin O-glucoside, cZROG = cis-zeatin riboside O-glucoside, cZ7G = cis-zeatin-7-glucoside, cZ9G = cis-zeatin-9-glucoside

CK re-supplementation assays

Due to the strong reduction of *tZ* observed earlier, I hypothesized that if reduced *tZ* availability was the reason for the reduction of *WUS* caused by TOR inhibition, then it should be possible to revert this negative effect by restoring *tZ* availability. Indeed, re-supplementation of 6-BA, *tZ*R and to a lesser extent also *tZ* restored the signal of the *WUS* reporter in TOR inhibitor treated seedlings (Fig. 9a). In contrast, equal amounts of *i*P, *cZ* or Kinetin derivatives did not significantly change the effect of AZD8055 on *WUS* expression. This suggested that the measured reduction of *tZ* after TOR inhibition was indeed causal for the inhibition of *WUS* expression. However, this result was somewhat surprising, since in etiolated seedlings I previously observed that 6-BA mediated induction of the *WUS* reporter was strongly reduced when applied together with AZD8055 (Fig. 4d). Because light grown seedlings produce photoassimilates, I reasoned that the increased sugar availability might explain the different outcomes between light and dark conditions. To this end, I assessed if supplementation of sucrose together with 6-BA or *tZ* would rescue the effect of AZD8055 on CK induced *WUS* expression in dark grown seedlings. Strikingly, when applied together with 6-BA sucrose supplementation rescued the inhibitory effect of AZD8055 to levels even higher than 6-BA alone (Fig. 9b). Similarly, sucrose also restored *WUS* levels after TOR inhibition when applied together with *tZ* (Fig. 9c). In sum, these observations suggested that TOR acts on *WUS* by controlling *tZ* availability and that sucrose together with *tZ* act as downstream effectors of TOR.

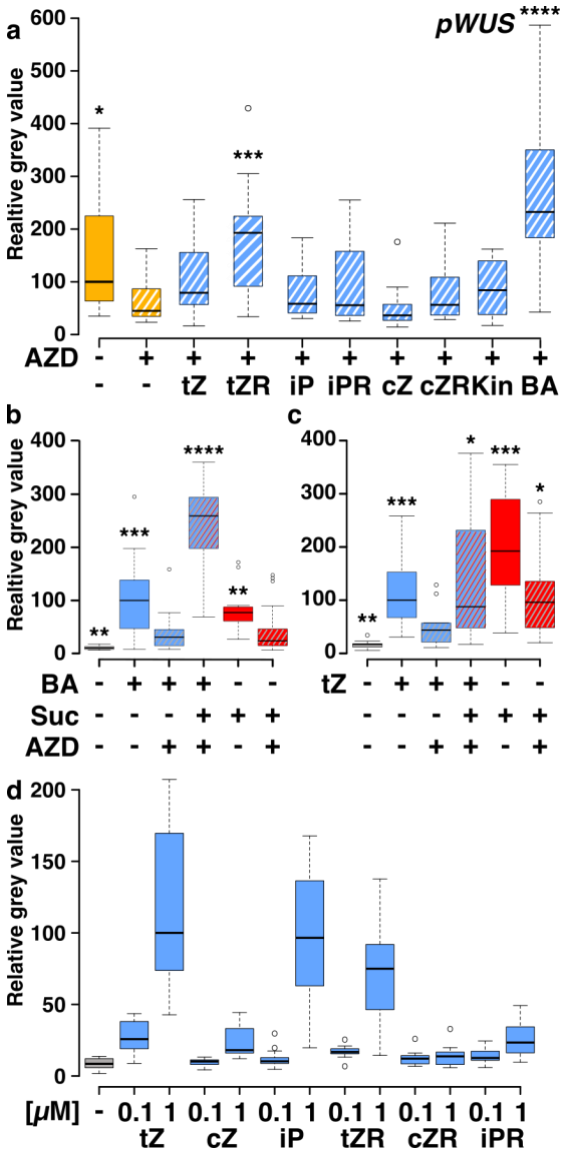


Figure 9: trans-Zeatin acts and sugars act downstream of TOR: a) Quantification of *pWUS:3xVenus:NLS* reporter signal. 3 day old seedlings grown in the light were treated with 2 µM AZD8055 and 0.5 µM of different CK derivatives for 1 day. Asterisks indicate significant differences compared to the AZD treated conditions. n=13-20. **b) + c)** Quantification of *pWUS:3xVenus:NLS* reporter signal. 2 day old etiolated seedlings were treated with 0.5 µM 6-benzyladenine (BA) or trans-zeatin (tZ), 2 µM AZD8055 and/or 1% sucrose for 3 days in the dark. Asterisks indicate significant differences compared to the BA+AZD or tZ+AZD condition. b) n=13-30 c) n=12-20. Significance levels were calculated using the Wilcoxon rank sum test with Hochberg correction (* = p < 0.05; ** = p < 0.01;*** = p < 0.001, **** = p < 0.0001). **d)** Quantification of *pWUS:3xVenus:NLS* reporter signal. 2 day old etiolated seedlings were treated with mock or the indicated concentrations of different CK derivatives for 3 days. tZ = trans-zeatin, tZR = trans-zeatin riboside, iP = isopentyladenine, iPR = isopentyladenosine, cZ = cis-zeatin, cZR = cis-zeatin riboside, Kin = kinetin, BA = 6-benzyladenine.

Reducing CKX activity changes the response to AZD8055

My results so far demonstrated that TOR controlled *WUS* by modulating *tZ* levels, but it remained unclear how this was achieved. A reduction of *tZ* levels is obtained either by attenuated biosynthesis or by increased turnover, and the previous observations justified both mechanisms. I decided to first assess the contribution of CYTOKININ OXIDASE/ DEHYDROGENASEs (CKXs), which are catabolic enzymes catalyzing the irreversible degradation of CKs. To this end, I subjected several *ckx* mutant lines to a physiological assay and monitored their shoot fresh weight in response to increasing concentrations of AZD8055. The obtained dose response curves revealed clear differences between some of the *ckx* mutant lines and wild type seedlings (Fig. 10a + e). Particularly, *ckx6*, *ckx5*, *ckx4* and *ckx3* showed a delayed response to AZD8055 compared with the wild type. Statistical testing confirmed this visual impression, as mixed ANOVA revealed significant contribution of the genotype on

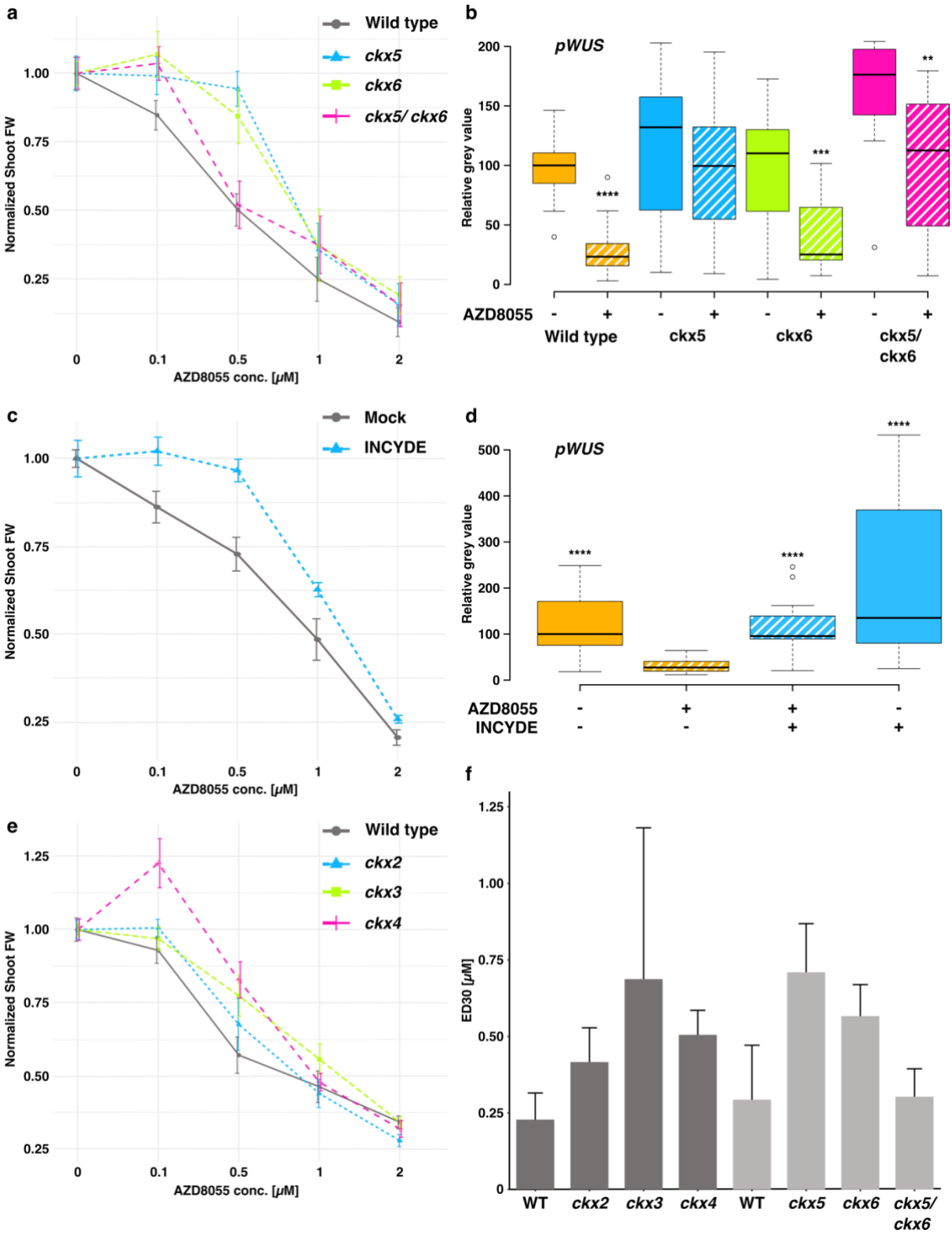
shoot fresh weight when tested with the interaction term Freshweight ~ AZDconentration*Genotype + (1|Experiment). Post-hoc pairwise comparisons showed that shoot fresh weight for wild type but also for *ckx2* and *ckx5/ckx6* seedlings was significantly different between 0 and 0.5 µM AZD8055 (Figure S3 – S5). In contrast, these comparisons were not significant for *ckx6*, *ckx5*, *ckx4* and *ckx3* seedlings suggesting that no growth inhibition occurred in these mutants up to 0.5 µM AZD8055. This was corroborated by the ED30 values which were up to three-fold higher for the *ckx* mutants than for the wild type (Fig. 10f). Only the *ckx5/ckx6* double mutant did not show an elevated ED30 value. These results suggested that mutations in CKX enzymes reduced the sensitivity towards TOR inhibition.

I also tested whether *WUS* response towards AZD8055 would be affected in *ckx* mutant lines. Thus, I analyzed expression of the *WUS* reporter in the background of *ckx5*, *ckx6* and *ckx5/ckx6* mutants. Strikingly, the inhibitory effect of AZD8055 on *WUS* expression was fully rescued in the *ckx5* mutant (Fig. 10b). In contrast, *WUS* expression was still repressed in *ckx6* mutant seedlings while in *ckx5/ckx6* double mutants I observed an additive effect of both single mutants. There, *WUS* levels were still reduced in response to TOR inhibitor treatment but were not significantly different from wild type mock levels. This demonstrated that CKX5 mediates the effect of TOR on *WUS* expression.

To further substantiate the results obtained with the *ckx* mutants I analyzed whether pharmacological inhibition of CKX enzymatic activity led to comparable outcomes. I utilized an active site inhibitor called INCYDE that globally reduces CKX activity and thus CK turnover (Berková et al., 2020; Zatloukal et al., 2008). The dose response curve from INCYDE treated seedlings phenocopied the *ckx5* mutant as no significant growth inhibition up to 0.5 µM AZD8055 occurred (Fig. 10c + Fig. S4). Consistently, *WUS* reporter expression was fully rescued when AZD8055 was supplied together INCYDE (Fig. 10d). These findings corroborated the observations made with the *ckx* mutants and supported the notion that CKX activity was repressed by active TOR and further implied a molecular interaction between TOR and CKX enzymes. However, my data also suggested that not all CKXs contributed equally, neither towards TOR mediated growth regulation nor towards TOR mediated *WUS* expression. Moreover, the role of specific CKXs appeared different regarding their effect on *WUS* or shoot growth. While the *ckx6* mutation reduced growth inhibition by AZD8055 but did not affect WUS, the *ckx5/ckx6* double mutant showed the opposite behavior. Additionally, *ckx* mutations provided resistance in the growth assay only up to 0.5 µM AZD8055 while *WUS* expression was rescued even at 2 µM AZD8055. These results suggested that TOR does not

regulate shoot growth solely through CK mediated *WUS* expression but also influences other cellular process relevant for growth control, although full TOR inhibition might also be a physiologically rather rare state.

Figure 10: Reduced CK turnover confers resistance towards TOR inhibition: a, c, e) Average shoot fresh weight per seedling was calculated from measurements of ~40 seedlings in batches of 5-10 for each measurement, that were transferred at 4 DAG to different concentrations of AZD8055 for 7 days. Data was pooled from two independent experiments and normalized to untreated average. Error bars represent standard error. Significant interaction was tested before normalization with ANOVA linear mixed effect models (Freshweight ~ AZDconc.*Genotype + (1|Experiment) for a) p = 0.00014 e) p = 6.87e-08 and for c) p = 0.0123. Pairwise comparisons are found in the supplements **a)** *ckx5*, *ckx6* and *ckx5/6* are in the genetic background of *pWUS:3xVenusNLS*, *pCLV3:mCherry:NLS* and were tested against this background. **c)** 75 nM INCYDE. **b, d)** Quantification of *pWUS:3xVenus:NLS* reporter signal. Seedlings were grown in the light for 3 days and treated with mock, 2 µM AZD8055 and/ or 75 nM INCYDE for 1 day. **b**) Asterisks indicate significant differences between the AZD treated condition and the respective Mock. No significant difference was found between untreated wt and treated *ckx5/6* mutant. n=15-29 **d)** Asterisks indicate significant differences compared to the AZD treated condition. n=16-19 f) ED30 values for different CKX mutant lines. Colors indicate different genetic background. Dark grey = col-0; light grey = *pWUS:3xVenusNLS*, *pCLV3:mCherry:NLS*. Significance levels were calculated using the Wilcoxon rank sum test with Hochberg correction (* = $p < 0.05$; ** = $p < 0.01$;*** = $p < 0.001$, **** = $p < 0.0001$).



TOR controls CKX1 protein abundance

The previous results pointed towards a repressive interaction between TOR and CKX activity, but the transcriptome data harbored no evidence for elevated expression of CKX transcripts in response to TOR inhibition. Thus, I monitored the expression levels of all seven CKX

isoforms in the shoot derived RNA isolated for RNAseq this time by qRT-PCR and found that only *CKX6* transcript levels were slightly elevated, whereas *CKX5* and *CKX2* transcripts appeared even reduced (Fig. 11). However, the confidence intervals for the fold changes were quite large, indicating substantial variation between the replicates even though the effects for *CKX2*, *CKX5* and *CKX6* were significant. These results could not explain the previously observed effects since the *ckx5* mutant was resistant to TOR which would have required elevated transcription. Thus, I concluded that TOR most likely regulates CKXs by a post-transcriptional mechanism.

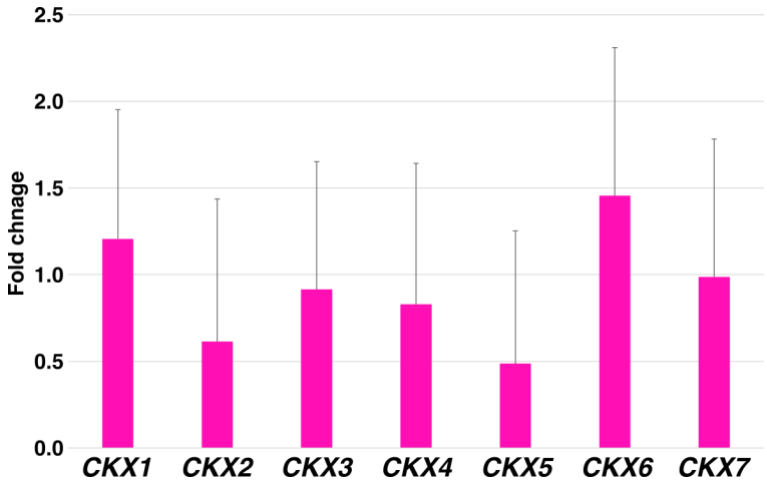


Figure 11: Transcriptional response of CKX genes towards TOR inhibition: q-RT-PCR of RNA from AZD8055 treated shoots of the RNAseq experiment. Expression was normalized to PP2A expression and the fold change of the AZD8055 treated samples was calculated relative to the mock. Error bars represent confidence interval of 3 biological replicates. Students t-test reveals significant differences for CKX2 (p = 0.015), CKX5 (p =0.031) and CKX6 (p = 0.045).

To this end, I tested the effect of TOR inhibition on protein levels of a constitutively expressed cMyc tagged CKX1 protein fusion (p35S:cMyc-CKX1) (Niemann et al., 2018). Western blots revealed that cMyc-CKX1 accumulated in protein extracts from AZD8055 treated seedling shoots (Fig. 12a). The effect size differed between experimental repetitions and ranged between 30 to 50 % increased protein levels but was consistent. In contrast, no significant change in the transcript levels was observed (Fig. 12b), thus indicating that the protein accumulation was either caused by a reduction of protein turnover or by enhanced translational efficiency.

To determine the protein half-life of cMyc-CKX1, I utilized the translational elongation inhibitor cycloheximide (CHX). After pre-incubation on AZD8055 or mock medium, seedlings were flooded with CHX solution and the protein decay over the course of 8 h was monitored in a western blot. The assay revealed no difference in cMyc-CKX1 protein stability between mock and AZD8055 treated shoots, indicated by the very similar slopes between the

different regression lines (Fig. 12c). In addition, the protein half-life was determined to about 4 h which was in agreement with previously published data (Niemann et al., 2018), validating my observation. The cMyc-CKX1 protein characterization revealed that TOR inhibition caused protein accumulation without affecting the turnover of the protein. Thus, all my data pointed towards enhanced translation of CKX1.

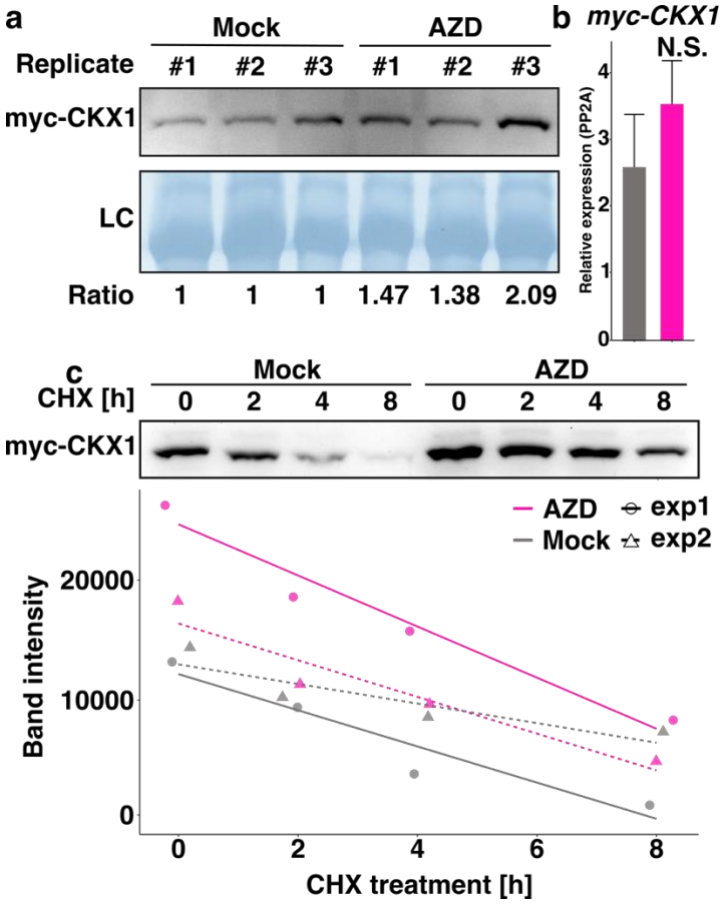


Figure 12: TOR regulates protein accumulation of myc-CKX1: a) Western blot of *p35S:cMyc-CKX1* 4 day old seedlings treated with either mock or 2µM AZD8055 for 8h. Shoot protein extract was probed with anti-cMyc serum. Replicates are from three independent experimental repetitions. Loading control (LC) stained with amido black. Ratios were calculated between band intensities from myc-CKX1 signal and the LC and normalized against the respective mock sample. **b)** q-RT-PCR of p35S:cMyc-CKX1 shoots with primers against the ectopic transcript. No significant difference using two biological replicates was found using paired t-test (p=0.3). Error bars represent standard deviation. **c)** Western blot of p35S:cMyc-CKX1 shoot protein extract probed with anti cMyc serum. Seedlings were pre-incubated on AZD8055 and then flooded with 200 µM cycloheximide solution for the indicated time. Band intensities from two independent experimental repetitions were plotted with linear regression lines.

TOR regulates translational efficiency of specific CKX transcripts

The analysis of CKX transcripts and protein, together with the physiological characterization of *ckx* mutants pointed towards a mechanism that involved translational regulation of CKX1 and potentially also other CKX isoforms. To test this directly, I performed a ribosome

fractionation experiment, separating heavy polysomes (fractions #1 - #3) from light polysomes (fractions #4 + #5) and monosomes (fractions #6 - #8) on a sucrose density gradient. Transcript quantification of all CKX isoforms revealed accumulation of several transcripts with translationally active heavy polysomes after TOR inhibitor treatment (Fig. 13). The strongest effect was observed for CKX1 transcripts, which showed up to five-fold enrichment in heavy polysome fractions, which was consistent with the observed accumulation of cMyc-CKX1 protein (Fig. 12a). In parallel, *CKX1* was strongly depleted from monosome fraction #8 indicating a shift from monosomes to polysomes. Similar effects were observed for *CKX3* and *CKX5* transcripts. Particularly *CKX3* transcripts were strongly accumulating in heavy polysome fractions (Fig. 13), consistent with the delayed response towards TOR inhibition of *ckx3* mutants in the physiological assay (Fig. 10e +f). However, *CKX3* transcripts were detected only in one out of three replicates, which can be explained by its narrow expression domain in the SAM (Bartrina et al., 2011; Werner et al., 2003). *CKX5* was consistently detected and accumulated in polysome fractions but the effect was not as pronounced compared with *CKX1*, albeit consistent (Fig. 13 + Fig. S6). This agreed with the observed resistance of *ckx5* mutants towards AZD8055 in the physiological assay and the rescue of *WUS* (Fig. 10a + b). While *ckx4* mutants also showed resistance in physiological analysis, the association with polysomes after TOR inhibition turned out ambiguous (Fig. 13). *CKX4* transcripts behaved contrary between experimental repetitions showing strong depletion from polysomes in one experiment while accumulating in another (Figure S6). In contrast with all other CKX isoforms, only the 5'-UTR leader sequence of *CKX4* harbored an upstream open reading frame (uORF) that could potentially repress *CKX4* main ORF translation. The *ckx6* mutant also showed altered behavior towards AZD8055 but had no effect on *WUS* (Fig. 10a + b). *CKX6* transcript showed only very subtle accumulation in one experiment in light polysome fraction #4 (Fig. 13) but had slightly increased expression levels as mentioned earlier (Fig. 11). No notable difference in polysome association was observed for *CKX2* and *CKX7* which was in line with the unchanged behavior of *ckx2* mutants in physiological assays (Fig. 10e). In conclusion, my results showed that association with efficiently translating polysomes of several CKX isoforms is repressed by active TOR. Particularly, *CKX1*, *CKX3* and *CKX5* translation was enhanced when TOR was inhibited, resulting in higher protein abundance as shown for cMyc-CKX1. These effects were consistent with the behavior of the respective mutants in AZD8055 response assays. No clear conclusion could be drawn for *CKX4* and *CKX6* isoforms. Even though the mutants show some resistance to AZD8055 treatment, the polysome fractionation assay allowed no clear

conclusions about the underlying mechanism. However, *CKX6* transcription appeared to be generally induced by TOR inhibition which could explain the resistance phenotype. The observed resistance of *ckx4* mutants was enigmatic. While the strong uORF contained in its 5'-leader sequence predicted it to be translationally repressed when TOR is inhibited, the polysome data did not consistently demonstrate translational repression. It can be speculated that the globally decreased CKX activity in *ckx4* mutants pleiotropically reduced the inhibitory effect of AZD8055 even though no specific interaction between TOR and CKX4 exists. However, the results for the CKXs clearly demonstrated that TOR represses translation of CKX1, CKX3 and CKX5 and thereby controls *tZ* levels and consequently *WUS* expression and shoot development.

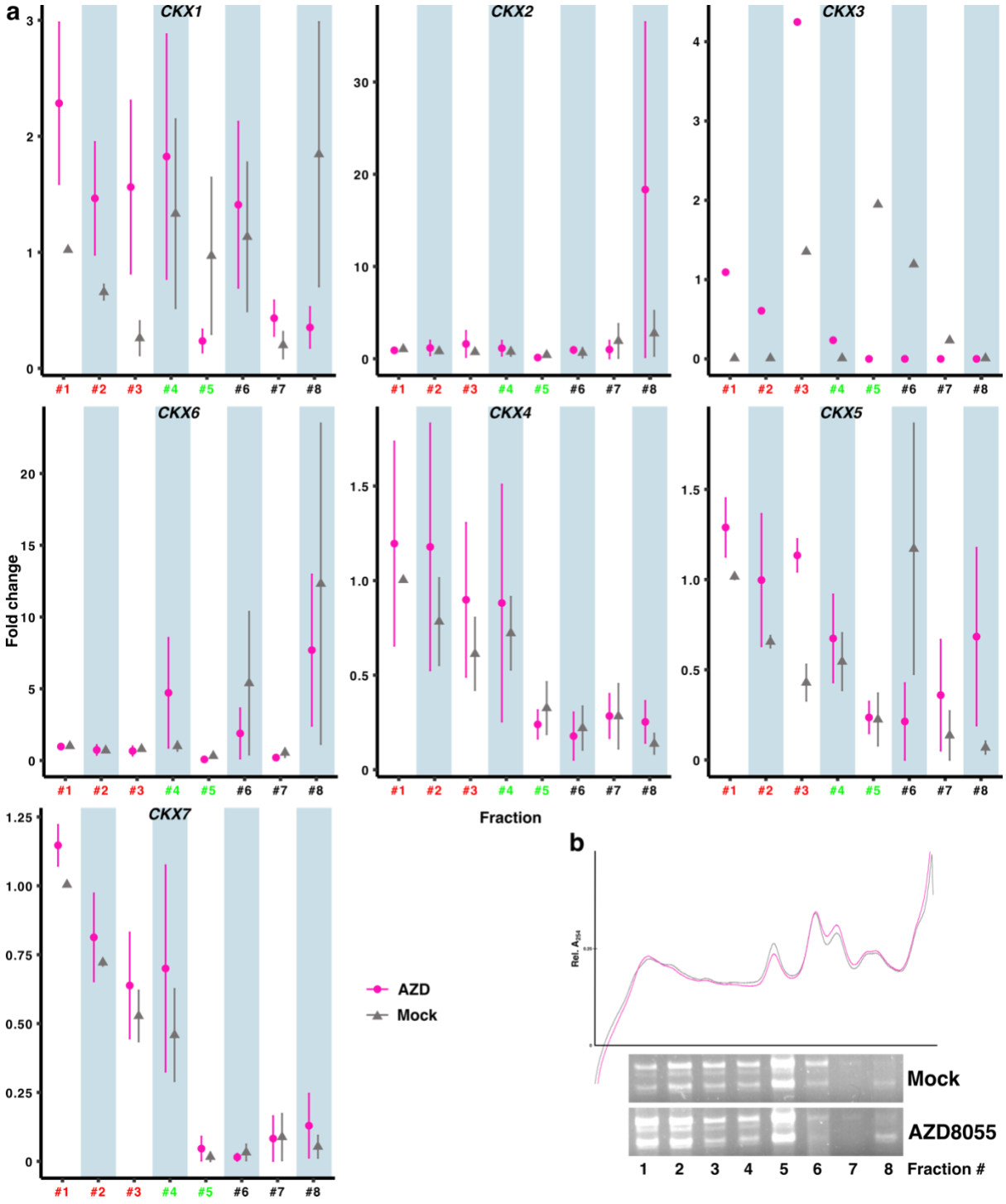


Figure 13: TOR regulates translational efficiency of specific CKX isoforms: a) CKX transcripts were quantified in ribosomal fractions by q-RT-PCR relative to *UBI10* and normalized to the respective mock of fraction #1. Error bars represent standard error of the mean of data pooled from three independent experimental repetitions (except CKX2+7 were only detected in 2 replicates, CKX3 was detected in 1 replicate). Red numbers indicate heavy polysomal fractions (fractions 1-3), green numbers light polysomal fractions (fractions 4+5) and black number monosomal fractions (fractions 6-8). Individual data points are shown in the supplements (Fig. S6). b) Representative absorption spectrum and RNA gel blot from polysome fractionation. Data for this experiment was jointly produced with Yihan Dong.

CK mediated effects of TOR on root growth

To this point, it became evident that TOR controlled shoot development by modulating the availability of *tZ* through translational control of CKX catabolic enzymes. However, TOR inhibition does also reduce root growth and since my previous data suggested that CK signaling was de-regulated also in roots upon AZD8055 treatment (Fig. 4b), I sought to shed light on how CK signaling contributes to TOR mediated root growth control. The strong increase in CK signaling implied by the elevated TCS reporter signal, suggested that TOR regulates root growth through repression of CK signaling (Fig. 4c). However, CK metabolic profiling revealed that the relevant CK metabolites were depleted after TOR inhibition (Fig. 8a - d), suggesting that the roots sensitivity towards CK was increased. This notion was supported by the transcriptional induction of AHKs, AHPs and type-B ARRs in my RNAseq dataset (Fig. 4b). Another important regulator of CK signaling is the pseudo-phosphotransferase AHP6, which is an atypical member of the AHP family and in contrast to the other members acts as a negative regulator of CK signaling through inhibition of phosphor transfer between AHKs and AHPs and has been previously characterized as a central regulator of xylem development in roots (Mähönen et al., 2006). I assayed the effect of AZD8055 treatment on a pAHP6:AHP6-Venus translational reporter line and found that after 24 h, Venus signal almost completely vanished from the root vasculature (Fig. 14), compared with mock treated roots that exhibited the usual protoxylem pattern of AHP6-Venus signal. The root transcriptome harbored no hints for transcriptional regulation of *AHP6*, suggesting that a post transcriptional mechanism could be responsible for the observed reduction in AHP6-Venus signal. Hence, I investigated whether expression of a pAHP6:GFP transcriptional reporter would have been similarly affected by AZD8055 treatment. Strikingly, there were no signs of reduced AHP6 expression (Fig. 14), indicating that indeed a post-transcriptional mechanism was involved. Inspection of the AHP6 5'-UTR revealed the presence of a uORF, indicating that AHP6 translation is potentially controlled by TOR dependent uORF skipping.

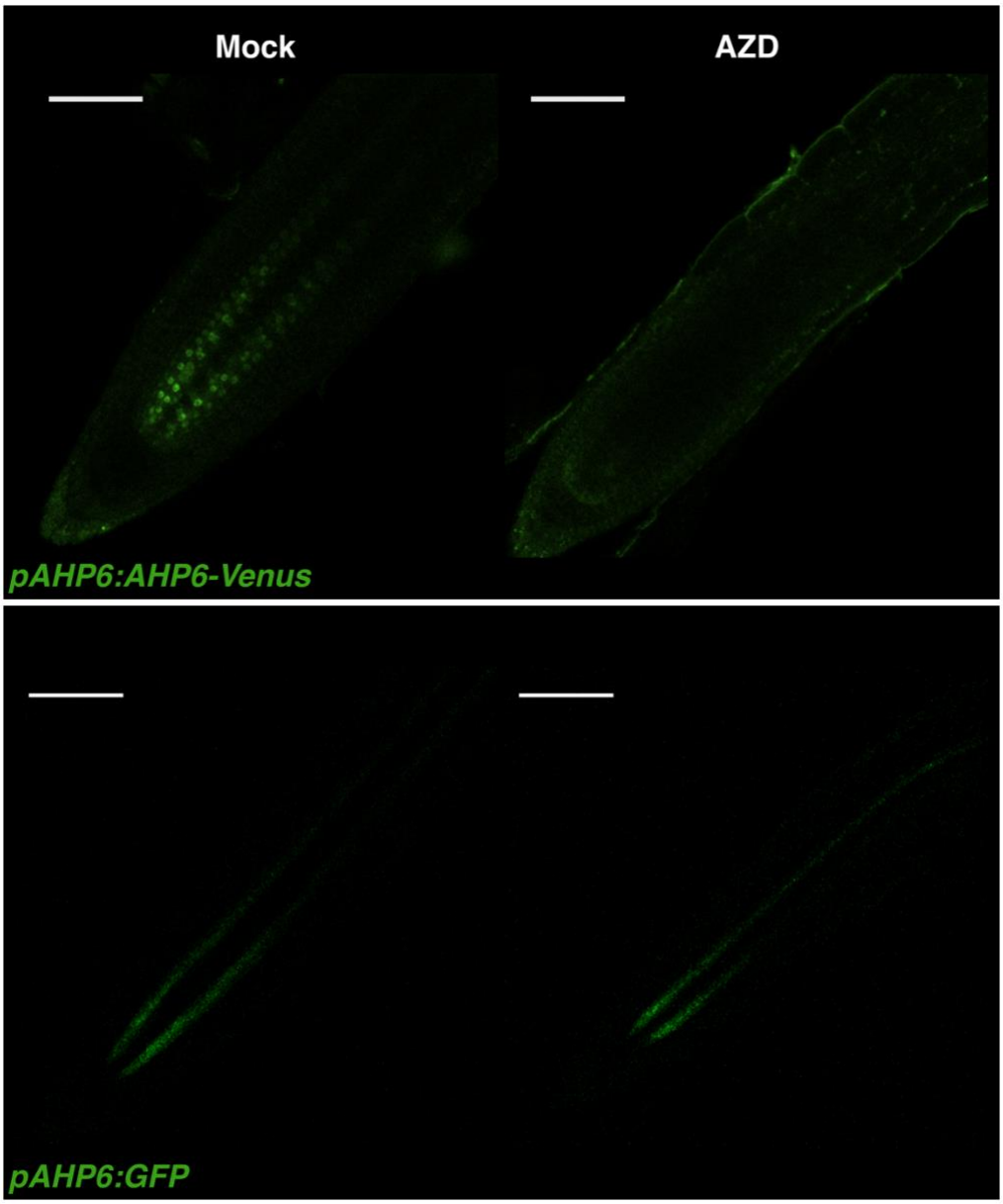


Figure 14: TOR controls AHP6 abundance: Upper panel: Representative confocal images of pAHp6:AHP6-Venus fusion line treated with 2 µM AZD8055 or DMSO for 24 h. Lower panel: Representative confocal images of the *pAHP6:GFP* transcriptional reporter treated with 2 µM AZD8055 or DMSO for 24 h.

Taken together, my observations supported a model in which TOR promotes root growth through bi-modal transcriptional- and translational repression of CK sensitivity. On the one hand through transcriptional repression of core CK signaling components and on the other hand by increasing translational efficiency of the CK negative regulator AHP6 in a uORF dependent manner.

Cytokinin as upstream regulator of TOR

So far, my data supported the notion of CK as a downstream mediator of TOR activity. However, several recent reports identified the plant hormones auxin and ABA as important

upstream activators and repressors of TOR respectively (Li et al., 2017; Schepetilnikov et al., 2017; Wang et al., 2018). CK is known to control cell proliferation and organ growth, wherefore I was intrigued whether CK might stimulate cell division by modulating TOR activity, as the ladder has been described to also control cell cycle progression. Thus, I monitored the effect of CK treatment on TOR activity in etiolated seedlings, which are characterized by low basal levels of TOR activity. Western blot analysis revealed a strong increase of S6K phosphorylation after treatment with 6-BA, indicating that CK stimulates TOR activity (Fig. 15a + b). Similar observations have been made for auxin and it has been shown that the GTPase ROP2 mediates auxin dependent TOR activation (Li et al., 2017; Schepetilnikov et al., 2017). I therefore wondered whether ROP2 also mediates CK dependent TOR activation. Indeed, in *rop2-1* mutant lines CK induced S6K phosphorylation was compromised compared to wild type plants (Fig. 14b). Similarly, expression of a dominant negative version of ROP2 (DN-ROP2) also impaired CK induced S6K phosphorylation (Fig. 15a). This suggested that CK stimulates TOR activity via ROP2. I hypothesized that if CK was an upstream regulator of TOR there should be a common downstream response between CK treatment and TOR inhibitor treatment. I thus compared my TOR inhibitor transcriptome with a publicly available CK meta transcriptome comprised of genes frequently de-regulated by CK treatment in multiple transcriptomic studies (Fig. 2d). Strikingly, around one third of the genes from both data sets was shared, indicating that one central aspect of CK action might be governed *via* TOR. I further asked whether TOR function was required for CK signaling and therefore investigated the effect of DN-ROP2 expression in a CK response assay (Schepetilnikov et al., 2017). I measured the expression levels of *ARR5* and *ARR6* in wild type seedlings and in seedlings expressing either wild type ROP2 or the dominant negative allele DN-ROP2 in an estradiol inducible manner. No difference occurred between ROP2 and wild type seedlings while DN-ROP2 expressing seedlings had already slightly lower initial expression levels for *ARR5* even without estradiol treatment (Fig. 15c), indicating potential leaky expression of the construct. However, *ARR6* appeared not affected without estradiol induction but substantially decreased following estradiol treatment and to a lesser extent also *ARR5*. This suggested that TOR inhibition via DN-ROP2 compromised CK response. However, it is difficult to differentiate between the effects of DN-ROP2 on TOR and TOR-independent effects.

In sum, my results suggested that CK stimulates TOR activity and that ROP2 mediates this activation similar to auxin induced TOR activation. The transcriptomic comparisons further suggested that an integral part of CK action is to stimulate TOR activity indicated by the large

overlap of target genes of both pathways but also by the shared functionality regarding control of growth and proliferation.

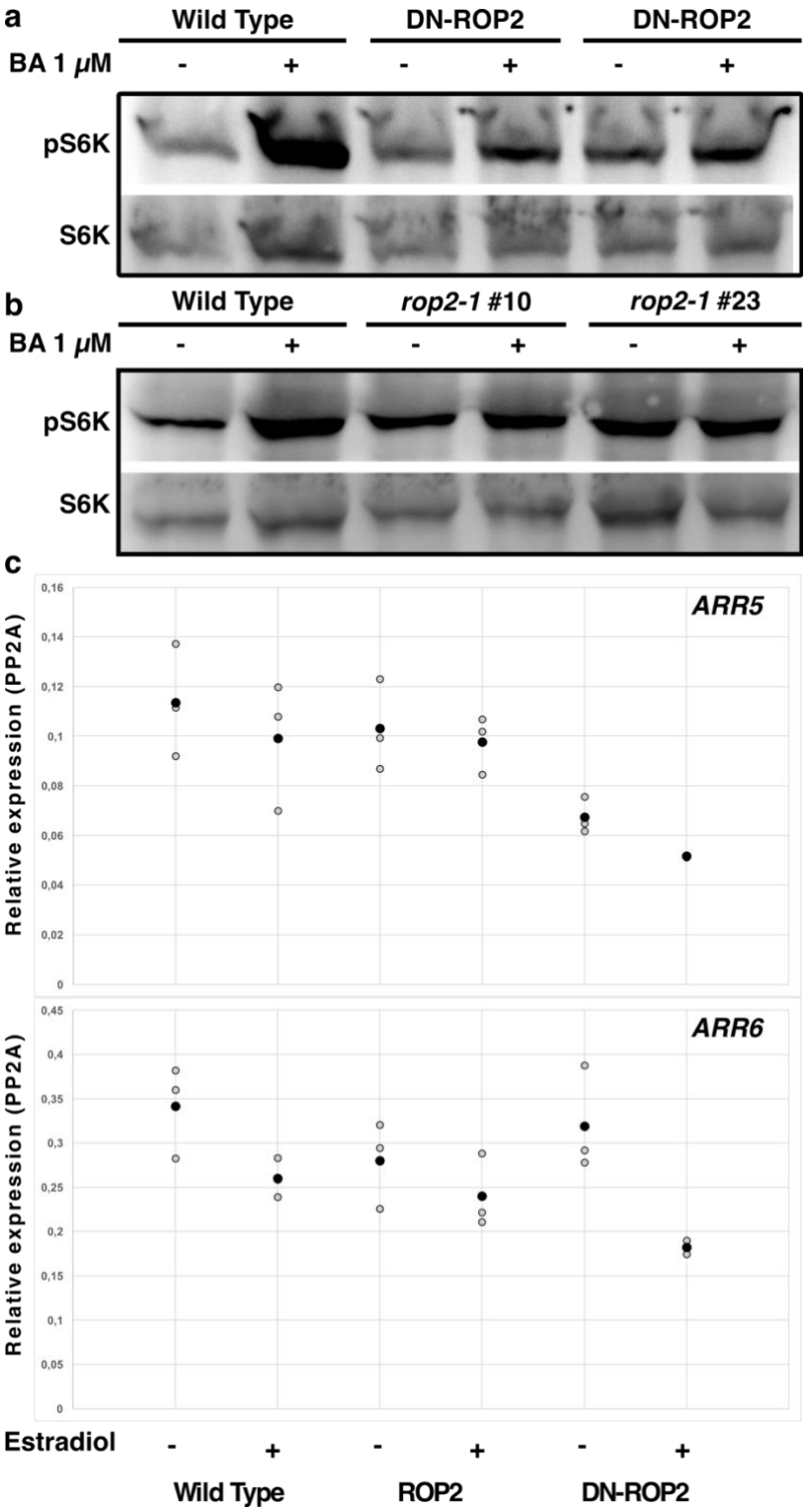


Figure 15: CK stimulates TOR activity via ROP2: a) + b) Western blots of protein extract obtained from 3 day old etiolated seedlings treated with 1 µM 6-BA for 24 h. Membranes were probed with anti phosphoS6K(T449) or anti S6K1/2 serum. Replicates are from independent seed batches **c)** qRT-PCR of RNA from 4 day old seedlings pre-treated with 10 µM estradiol for 24 h and sprayed with 0.5 µM 6-BA for 30 min. Seedlings were either wild type or estradiol inducible wild type ROP2 or dominant negative ROP2 (DN-ROP2). Grey dots are replicates, black dots are the calculated mean.

raptor1b heterografts suggest a systemic component of the TOR complex

My previous observations implied a function for CK both upstream and downstream of TOR. Since the investigated tissues were heterogenous and comprised of cells with variable

differentiation states, this raised the question whether TOR might have different functions in different cell types or might differ among cells with variable degrees of differentiation. While early TOR expression studies suggested that TOR is predominantly expressed in meristematic tissues and callus (Menand et al., 2002), a recent report detected higher TOR activity in differentiated source tissues compared with growing sink tissues (Brunkard et al., 2020), corroborating the hypothesis for different roles of TOR. Other evidence comes from the observation that dominant active PHYTOCHROME B (phyB) is only able to induce *WUS* when expressed outside of the SAM (Pfeiffer et al., 2016). Together with my finding that TOR controls *tZ* availability which acts as a systemic long-range signal within the plant, I hypothesized that TOR might systemically contribute to control plant growth and development. To test this, I utilized *raptor1b* mutants which are devoid of a regulatory TOR subunit and hence are impaired in TOR function. I grafted *raptor1b* shoots on wild type roots and *vice versa* and generated wild type and *raptor1b* homografts as control. Rosette growth of *raptor1b* homograft was strongly impaired compared with wild type homograft (Fig. 16a). In contrast, rosettes of WT/*raptor1b* and *raptor1b*/WT heterografts grew at a comparable pace compared with the wild type homograft. Additionally, the *raptor1b* homograft was strongly delayed in the transition to flowering, as no bolting occurred when the wild type homograft had already developed a full inflorescence (Fig. 16b - d). The wild type shoot scion showed no delayed flowering when grafted with a *raptor1b* root, while the *raptor1b* shoots were substantially delayed even when grafted on a wild type root. However, wild type roots partially rescued the delayed flowering phenotype of the *raptor1b* shoot scion, as initial bolts were visible when the *raptor1b* homograft still showed no signs of bolting (Fig. 16 c + d).

The observation that the developmental delay and even more so the slower growth phenotype of *raptor1b* mutant shoot was rescued by grafting on a wild type root suggested that there is indeed a mobile, systemically active compound emitted from roots that depends on root derived TOR activity. However, the fact that wild type shoot development does not seem to be affected when grafted on *raptor1b* roots indicated that shoots can compensate for the loss of such root derived compounds. The nature of the mobile compound remains obscure, but *tZ* is certainly one of the most promising candidates. *tZ* is known to be mostly produced in roots under normal conditions by CYP735A1/A2. However, loss of root derived *tZ* in the *cyp735a1/a2* double mutant is compensated by wild type shoots in grafting experiments (Kiba et al., 2013). Similar to my results, *cyp735a1/a2* mutant shoots are rescued when grafted on wild type roots. Moreover, the delayed shoot development and delayed flowering transition are both phenotypes known to be strongly influenced by root derived *tZ* (D'Aloia et al., 2011;

Landrein et al., 2018; Osugi et al., 2017). Together with the strong *t*Z depletion observed after TOR inhibition it is thus very likely that *t*Z is the causal agent for TOR systemic effects. However, at this stage I cannot rule out the possibility that other compounds contribute to the systemic behavior.

Figure 16: Systemic rescue of raptor1b mutant shoots: Pictures of homo- and heterografts between wild type and raptor1b seedlings. Nomenclature is [*shoot donor/root donor*]. a) Representative top down images of homo- and heterograft 14 days post grafting. b) All grafts from one experiment 21 days post grafting. c) + d) Side views of grafts from b). Grafts in c) show emerging bolts while no bolting is seen in d).

Autophagy is inversely regulated in shoot versus roots

As mentioned earlier my transcriptome data harbored many hints to other TOR related pathways and autophagy caught my special attention as many autophagy related genes were induced in roots after AZD8055 treatment but repressed in shoots. While TOR dependent regulation of autophagy is well characterized, the repression of many ATG genes (ATG3, ATG6, ATG8C, ATG8D, ATG8H, ATG8G, ATG13, ATG18G, ATG18H) in shoots was puzzling as it suggested that autophagy might be attenuated. Since TOR is a known repressor of autophagy throughout all eucaryotes and AZD8055 together with other TOR inhibitors is commonly utilized in studies related to autophagy to induce autophagy (Dauphinee et al., 2019; Dong et al., 2015; Liu & Sabatini, 2020; Pu et al., 2017), this observation prompted me to investigate whether TOR dependent autophagy regulation was different in shoots. To this end, I performed a GFP-ATG8 cleavage assay, monitoring the turnover of GFP-ATG8 in response to AZD8055. The ratio between GFP-ATG8 and free GFP serves as a read out for autophagic flux as GFP-ATG8 is incorporated in all autophagosomes and subsequently transported to lytic vacuoles where ATG8 is degraded but GFP accumulates. Western blot analysis of AZD8055 treated p35:GFP-ATG8 plants revealed that indeed no accumulation of free GFP occurred 8 h after treatment in shoots (Fig. 17a), while in roots substantial accumulation of free GFP was observed concomitant with a reduction of GFP-ATG8 fusion protein. This suggested that AZD8055 treatment as expected induced autophagy in roots but did not induce autophagy in shoots as predicted by my RNAseq data. No accumulation of free GFP was observed in an *atg5* mutant background lacking the central inducer of autophagy ATG5 confirming that this was indeed an autophagy specific process (Fig. 17b).

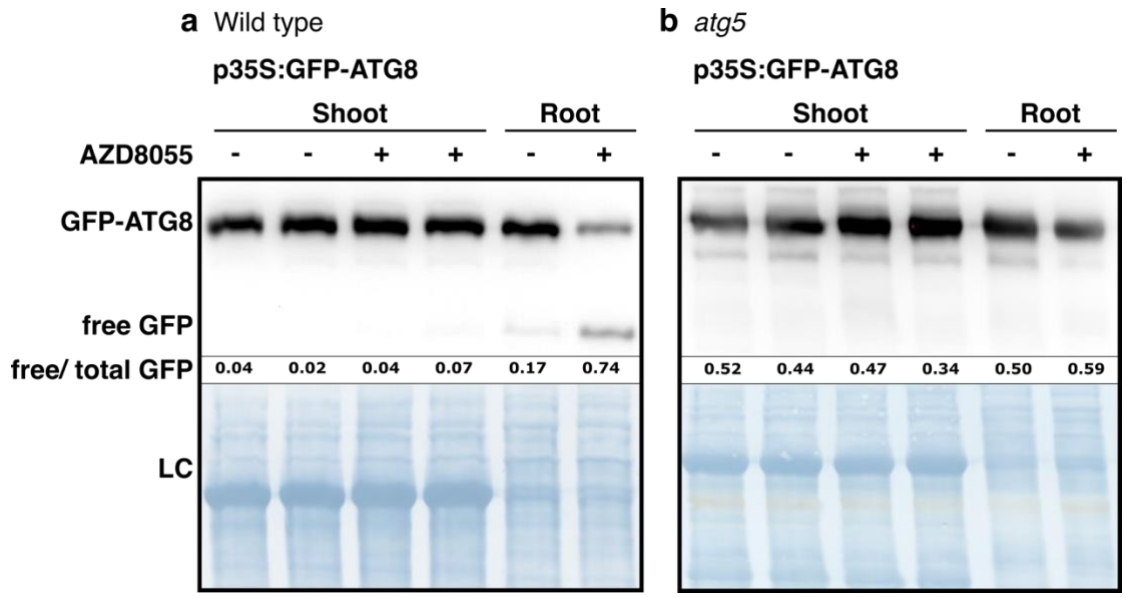


Figure 17: Differential regulation of autophagy in roots and shoots: a) + b) Western blots of protein extract obtained from 4 day old seedlings treated with 2µM AZD8055 or DMSO for 8 h. Membranes were probed with anti GFP serum. Ratios were calculated between the intensities measured for the upper GFP-ATG8 band and the lower free GFP band. LC = Loading control (amido black staining). a) p35S:GFP-ATG8 in wild type background b) p35S:GFP-ATG8 in *atg5* mutant background.

Discussion

Phenotypic plasticity is what allows plants to thrive in a wide range of habitats and successfully adapt to dynamic environments. Pluripotent stem cell systems that are maintained throughout a plants whole lifecycle grant plants the flexibility to adjust their growth and development to maximize the chances of successful reproduction even under suboptimal conditions. This implies that intricate sensing- and signaling mechanisms must be at play and instruct stem cell behavior to generate favorable cellular pattern. Resolving the underlying molecular mechanisms will help us to understand the evolutionary success of plants and might ultimately facilitate our efforts to generate resilient and productive agroecosystems. It has been shown before that light and nutrient signaling pathways converge to stimulate TOR kinase activity which integrates these signals to control expression of *WUS*, a master regulator of stem cell homeostasis. However, it remained obscure how TOR activity is conveyed to modulate *WUS* promoter activity. Thus, the underlying work aimed to identify downstream effectors of the TOR kinase that control *WUS* expression and plant development.

In an RNAseq based approach I utilized three different pharmacological inhibitors of TOR kinase activity and generated a list of high confidence TOR target genes. From there, I found that CK signaling was deregulated in the SAM and that TOR inhibition generally interferes with CK functional output. While CK sensing and signaling appeared still functional, the levels of several CK metabolites were drastically changed. I showed that TOR influences *WUS* expression levels by controlling the abundance of *tZ* derivatives and that *tZ* together with sucrose acts downstream of TOR. Based on resistance of CKX mutants towards TOR inhibition in physiological assays and WUS reporter studies, it turned out that TOR limits catabolic turnover of *tZ*. Mechanistically, this is achieved by controlling CKX protein accumulation through translational repression of several CKX mRNAs.

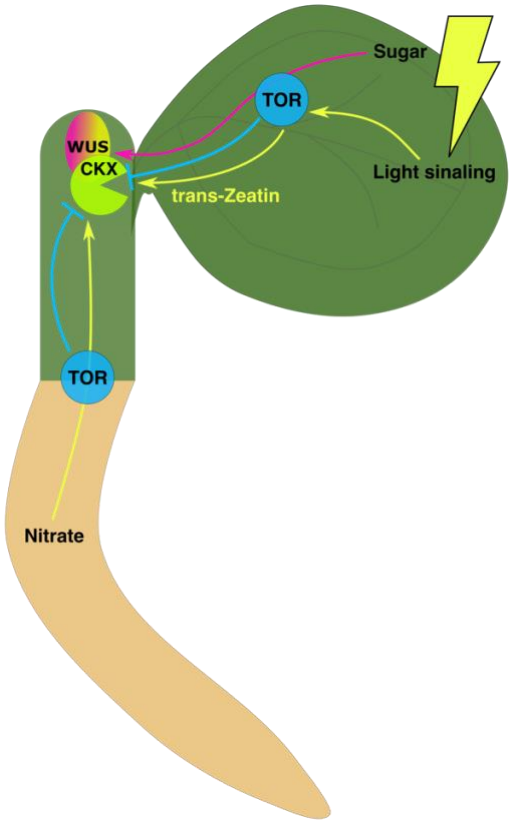


Figure 18: TOR controls SAM homeostasis Schematic drawing of TOR function as integrator of environmental signals. Light signaling pathways, photoassimilates and nitrate stimulate TOR activity thereby limiting CKX protein abundance and thus turnover of *trans*-Zeatin. *Trans*-Zeatin and sugar availability control *WUS* expression in the SAM downstream of TOR thereby tuning stem cell activity and organogenesis.

The TOR transcriptome

Transcriptome studies serve as an important resource to characterize the functional impact of biological entities and allow for the identification of novel molecular players. The prerequisite are transcriptomic datasets fulfilling high standards regarding study design and reproducibility. All currently available transcriptome studies investigating TOR dependent transcriptomic changes in plants analyze whole seedlings and utilize only one method to interfere with TOR activity (Caldana et al., 2013; P. Dong et al., 2015; Scarpin et al., 2020). Thus, my list of shoot specific TOR target genes provides the highest confidence dataset currently available, as only genes that are consistently differentially regulated in at least 6 biological replicates by at least two different compounds are considered. The high confidence is confirmed by the high predictability of this dataset. Primarily, my experiments confirmed the predicted impact of TOR inhibition on CK signaling. Furthermore, I identified several biological processes affected by TOR that have been published, some of which have just recently been reported and were completely unknown at the time the dataset was generated. For example, only recently connections between TOR and ER dependent UPR have been reported (Angelos & Brandizzi, 2021; Cao et al., 2019). Also, TOR dependent RNA methylation has only recently been discovered in mammalian systems (Cho et al., 2021; Tang et al., 2021) while my dataset hints towards a role for TOR in RNA methylation also in plants. Moreover, many Autophagy

related ATG genes were repressed among the shoot DEGs but induced in the root. This pointed towards different effects of AZD8055 treatment in shoots compared with roots which is rather surprising since it challenges the current notion of TOR as a general repressor of autophagy. However, I could confirm these observations in an ATG-GFP cleavage assay, further validating the predictive capacity of the underlying RNAseq dataset. These observations at the same time underpin the superiority of tissue specific profiling approaches, as I would have not been able to resolve inversely regulated genes as mentioned for autophagy but also UPR related genes. Obviously, the dataset still has limited resolution regarding specific cell types and differentiation states. Hence, it would be very interesting to benchmark my dataset against single cell sequencing datasets after TOR inhibitor treatment.

Most comments regarding the shoot- also apply to the root transcriptome. I decided to only sequence the AZD8055 treated roots, which resulted in lower confidence levels but instead offered higher sequencing depth. The GO analysis and the predictions based on the root data regarding enhanced CK signaling, induction of autophagy and UPR demonstrate sufficient reliability. However, it was puzzling to see photosynthesis related genes predominantly enriched among the downregulated genes. This is most likely a consequence of root illumination, as in my experimental setup seedlings are grown on transparent agar plates that expose all plant parts to light. There are recent developments towards experimental systems that prevent root illumination as it has been shown on several occasions that this can cause artificial molecular phenomena that would not occur in dark grown roots (Cabrera et al., 2021).

Interestingly, the overlap with other TOR inhibitor transcriptome datasets is not overwhelming, albeit significant. It is not surprising that the overlap with inducible amiTOR lines after 3 and 6 days is rather small (Caldana et al., 2013), as it can be anticipated that removing a scaffold protein from a presumable megadalton protein complex has much broader implications for cellular homeostasis compared with simply inhibiting phosphorylation of target genes. This observation points towards a much broader role of the TOR complex exceeding its function as a protein kinase. Observations of huge scTORC1 polymers, so called TOROIDS, in yeast and functions for TORC2 in regulating membrane curvature (Prouteau et al., 2017; Riggi et al., 2019, 2020) corroborate this notion and future work might reveal that the TOR complex is a central organizer of cellular structures determining structure of organelles and other compartments. Noteworthy, my AZD8055 dataset shares more DEGs with a dataset generated after 2h of TORIN2 (Scarpin et al., 2020) treatment compared with another dataset that also utilized AZD8055 but was generated 24 h after treatment (P. Dong et al., 2015). This indicates that differences in the duration of the treatment have a larger effect

on the divergence of the transcriptome than the actual substance. It is thus likely that the TOR transcriptional response is structured in a multilayered cascade, and it would be interesting to resolve these different transcriptional tiers in the future. Epigenetic components might play a role in the evolving transcriptional response as recent work implied a loss of H3K27me3 at genomic loci related to biotic stress upon TOR inhibition (Y. Dong et al., 2021).

TOR controls CK homeostasis

My RNAseq experiment revealed differential regulation of all major phytohormone signaling pathways which agrees with observations by other groups (Li et al., 2017; Song et al., 2017; Wang et al., 2018; Zhang et al., 2016). CK related genes caught my attention due to CKs well characterized role in SAM homeostasis and because no connection had been reported between CK and TOR. The whole CK signaling pathway appeared to be deregulated upon TOR inhibition and pointed towards reduced output of CK signaling in shoots and increased sensitivity of roots, which I confirmed with the *pTCSn:GUS* CK signaling reporter (Zürcher et al., 2013). Since TOR inhibition also abolished CK induced expression of *WUS,* I sought to identify where in the CK signaling pathway TOR interferes with. Several experiments confirmed that seedlings were in principle still responsive towards *i*P after TOR inhibition but showed almost no sensitivity towards *tZ* which led to the conclusion that exogenously added *tZ* must have been either degraded or sequestered. Consequently, I measured CK metabolites and found drastic changes in several compounds. Especially *tZ* derivatives, which are the most relevant CKs for shoot development and *WUS*, were strongly depleted, while *i*P active bases accumulated in shoots but were also depleted in roots. At the same time all *cZ* derivatives strongly accumulated, which was quite intriguing considering that *cZ* compounds originate from isopentylated tRNA turnover and TOR has been implicated to promote tRNA biogenesis (Shor et al., 2010). This observation suggests that TOR not only promotes tRNA biogenesis but might also actively represses tRNA turnover. Usually, tRNAs are thought to have a long half-life in the range of 2-3 days in eucaryotes (Kanerva et al., 1978; Karnahl & Wasternack, 1992; Nwagwu & Nana, 1980), however, my finding suggest that they might need to be actively stabilized by TOR activity and otherwise are rapidly turned over.

To explain the observed effects several scenarios are to be considered. *i*PRMP is synthesized from free AMP and further processed towards *tZ*RMP by CYP735A1/A2 enzymes. Depletion of *tZ* coupled with *i*P accumulation in shoots after TOR inhibition suggests that *i*P turnover might be reduced, particularly because *CYP735A2* transcripts were depleted in the transcriptome data. However, CYP735A1/A2 have almost no affinity towards free *i*P bases,

and the conversion is thought to happen almost exclusively on the precursor level (Takei et al., 2004). Consequently, also *i*PRMP precursors should accumulate but instead are also reduced after TOR inhibition. Since *IPT1* and *LOG5* and *LOG8* transcripts were induced, more *i*PRMP might be synthesized by IPT1 and more *i*PRMP might be converted towards free *i*P by LOGs, which would be an alternative hypothesis explaining *i*P accumulation. The results of my later experiments clearly pointed in the direction of increased turnover of *tZ* by CKX enzymes. It remains puzzling why *tZ* would be so efficiently degraded but iP not, as the affinities of most CKXs are not so drastically different between *tZ* and *i*P to sufficiently explain selective turnover. However, all CKX activity assays are done *in vitro* and no information about *in vivo* selectivity profiles is available. Another layer of complexity is added by the fact that the subcellular distribution of the different CK species remains unclear. It has just recently been demonstrated that CK receptors initiate signaling from the plasma membrane (PM) and from the ER membrane and characterization of the CK importer PUP14 suggests that signaling form the PM might be more important. Also, an ER importer ENT6 and a cellular exporter ABCG14 have been identified. In addition, the seven CKX family members also have different subcellular localization, although most of them are predicted to reside either in the ER membrane or the ER lumen and might also be secreted which has not been shown for *Arabidopsis*. Together, this demonstrates that CKs likely are distributed throughout many cellular compartments, and it can be thus speculated that selective transport might separate *i*P and *tZ* to different cellular compartments which might result in different turnover rates. But it could as well be that some CKX isoforms indeed have higher selectivity towards specific compounds *in vivo* which could also involve cofactors that determine specificity. Noteworthy, in some *in vitro* studies CKX1 indeed shows much higher affinity for *tZ* derivatives compared with *i*P (Kowalska et al., 2010), which is interesting since I found the strongest effect on CKX1 translational efficiency. Moreover, in *ckx3/ckx5* double mutants free *tZ* accumulates whereas free *i*P does not (Bartrina et al., 2011) and both transcripts accumulated in polysome fractions after TOR inhibition.

In conclusion, it appears that TOR inhibition induced pathway wide changes in CK homeostasis on transcripts of biosynthetic genes and signaling compounds but also on many CK metabolites. It is clear, that the reduced CK signaling output in shoots is caused by a reduction in tZ derivatives. However, at this stage it remains unresolved whether the transcriptional changes in CK biosynthetic enzymes contribute to the observed changes in CK metabolites or whether this might be rather a result of feedback regulation. CK profiling with *cyp735a1/a2* double mutants and several *ckx* mutants under TOR inhibition might shed light

on the individual contributions of these pathways in the future. However, even if catabolic turnover might not explain all observed metabolic changes in CK metabolites, the full rescue of *WUS* expression in the *ckx5* mutant and under INCYDE supplementation and the resistance conferred by several *ckx* mutants strongly support that increased *tZ* turnover contributes significantly.

Sucrose and *t*Z are downstream effectors of TOR

It appeared likely that the drastic reduction of *tZ* caused the detrimental effects of TOR inhibition on *WUS* and shoot development and indeed *tZR* and the synthetic CK 6-BA re-supplementation rescued *WUS* expression after TOR inhibitor treatment in light grown seedlings. This was somewhat puzzling as 6-BA induced *WUS* expression in etiolated seedlings was almost completely abolished when TOR was inhibited. I reasoned that availability of photoassimilates might explain the different outcomes and strikingly when sucrose was supplied together with either 6-BA or *tZ* *WUS* expression remained even higher compared with only CK even though TOR was inhibited. This was the first condition where TOR inhibition would not reduce *WUS* expression, clearly demonstrating that *tZ* together with sucrose acts downstream of TOR. The higher potential of 6-BA to rescue *WUS* compared with *tZ* or *tZR* correlates well with 6-BA being a poor substrate for CKXs (Frébortová et al., 2004). It is however surprising, that Kinetin was not able to rescue *WUS* since it is also not very efficiently degraded by CKXs. As mentioned earlier this as well could be a result of the different situation *in vivo* compared with in vitro activity studies and of differential subcellular distribution profiles. Compared with *iP* and *cZ*, *tZ* possess higher affinities towards CK receptors which explains why these molecules might not be able to rescue *WUS* (Romanov et al., 2006).

The synergistic effect between sucrose and CK indicates that sucrose is required to limit CK turnover by CKXs and is in line with the previously observed dependency of pTCSn:GUS signaling in the SAM on active photosynthesis (Pfeiffer et al., 2016). Sucrose is known to act as an activator of TOR whereas in could be argued that the presence of sucrose facilitates TOR mediated translational repression of CKXs. However, it is unclear why this would still work when TOR kinase activity is inhibited by AZD8055. It can be speculated that a TOR independent pathway could act in parallel, which is supported by my recent experiments showing that ammonium stress also leads to CKX1 protein accumulation, while not affecting TOR activity (own preliminary results; data not shown).

Mutations in several CKXs confer resistance to TOR inhibitor

While at this point it was clear that TOR controls *WUS* by promoting *tZ* accumulation, it remained unresolved whether impaired biosynthesis or enhanced turnover were causal for the reduction of *tZ*. To this end, I chose to investigate the contribution of catabolic *tZ* turnover and characterized shoot growth of several CKX mutant lines in response to increasing concentrations of TOR inhibitor. It turned out that *ckx3*, *ckx4*, *ckx5* and *ckx6* were resistant towards shoot growth inhibition up to 0.5 μM AZD8055. While *ckx2* and *ckx5*/*ckx6* double mutants had a slightly elevated ED30 value, statistical analysis of the growth curve did not reveal a significantly different response towards AZD8055. These results partially reflected the polysome profiling data where *CKX5* and *CKX3* transcripts were enriched among efficiently translating polysomes after TOR inhibition. However, the situation for *CKX4* and *CKX6* was different. *CKX6* transcripts exhibited no differences in polysome profiles, but instead were the only CKX transcripts with slightly elevated expression revealed by qRT-PCR. In contrast, no such effect was observed for *CKX4* transcripts. However, the results in the polysome experiment were ambiguous for *CKX4* as in some experimental repetitions it was strongly depleted from polysomes while it substantially accumulated in other replicates. The resistance towards TOR inhibition conferred by *ckx4* suggested a molecular interaction between TOR and CKX4, however, the reduced responsiveness could also be explained by globally reduced CK turnover in the *ckx4* mutant which pleiotropically leads to a reduced response towards TOR inhibition. More experimental repetitions, protein profiling by mass spec or translational reporter lines are necessary to answer whether TOR affects CKX4 protein levels. Intriguingly, *CKX4* is the only CKX isoform that harbors potential TOR dependent uORF in its 5'-UTR, which in consequence should result in impaired translational efficiency when TOR is inhibited, because TOR activity initiates uORF skipping and thereby favors translation of the main ORF. TOR might also regulate enzymatic activity of CKX4, for example by mediating changes in subcellular localization, post-translational modifications or co-factor availability. In addition to shoot growth, the *ckx5* mutation also fully rescued *WUS* expression upon TOR inhibition while *ckx6* had no effect. Intriguingly, both genes are expressed in the SAM of seedlings, which raises the question why the outcome is different, particularly because *ckx*6 seems to confer resistance towards TOR in the physiological assay. In addition, the *ckx5*/*ckx6* double mutant shows an additive behavior in the *WUS* reporter assay and while it still shows reduced *WUS* expression after TOR inhibition the levels are not reduced below the WT mock levels. Notably, *ckx5* fully rescued *WUS* even at 2 μM AZD8055 but conferred resistance in the growth assay only up to 0.5 μM AZD8055, similar to other *ckx* mutants. Previous work demonstrated a

strong correlation between *tZ* levels, CK signaling in the SAM, *WUS* expression and rosette growth suggesting that rosette growth scales with *tZ* availability and thus *WUS* expression. However, the partial inconsistencies between the full resistance of *ckx5* regarding *WUS* expression and the only partial resistance conferred in terms of shoot growth, demonstrate that TOR dependent growth regulation is a pleiotropic trait that only partially depends on *WUS* expression and stem cell activity. The different behavior of *ckx6* and *ckx5/ckx6* between *WUS* reporter assays and shoot growth assays, highlight the complex interaction between CK and cellular behavior in different cellular contexts. CK not only affects *WUS* expression and SAM homeostasis but also regulates growth and proliferation of pavement cells in leaves (Li et al., 2013; Skalák et al., 2019), another important factor that contributes to the complex trait shoot growth. It is thus remarkable that pharmacological inhibition of CKX activity with the active site inhibitor INCYDE conferred resistance towards growth inhibition and *WUS* repression caused by TOR inhibitor treatment and basically phenocopied the *ckx5* mutant. The physiological assays and *WUS* reporter analysis clearly demonstrated that TOR regulates *WUS* and to a lesser extent also shoot growth by modulating the activity of CKX catabolic enzymes and gave a strong hint towards a molecular interaction between TOR and CKXs.

TOR represses translation of CKXs

There were no hints in my transcriptome data that pointed towards transcriptional regulation of CKX enzymes even though the previous experiments suggested a regulatory interaction between TOR and CKX activity. Since CKXs are rather low abundant I performed an additional qRT-PCR experiment. Most CKX transcripts appeared indeed unaffected after TOR inhibition and *CKX2* and *CKX5* were even depleted while only *CKX6* showed a mild but significant transcript accumulation which could explain the resistance observed for *ckx6* towards AZD8055 but not for the other CKX mutants. Particularly *ckx5* showed consistent resistance throughout physiological and WUS reporter assays but had lower transcript levels. I thus concluded that the nature of the molecular interaction must involve a post-transcriptional mechanism. Therefore, I chose to analyze protein accumulation of a translational fusion of CKX1 and found consistently increased myc-CKX1 protein levels. Since there were no signs of altered protein stability in a cycloheximide chase experiment, I concluded that enhanced translation must have caused myc-CKX1 protein levels. Polysome fractionation confirmed that *CKX1*, *CKX3* and *CKX5* transcripts were enriched with translationally highly active polysomes. The strongest effect was observed for *CKX1* whereas *CKX3* was only detected in one of three biological replicates which is probably due to its narrow expression domain in the SAM. As mentioned above this was consistent with the CKX

mutant characterization. Even though there is no CKX1 mutant available it has been shown that CKX1 has a higher affinity towards *t*Z compared with *i*P whereas it is likely that also enhanced CKX1 translation contributes to the altered *t*Z homeostasis observed after CK profiling. The result was rather unexpected since TOR activity is well known to promote translation globally but also of specific transcripts via uORF skipping. My results demonstrate that TOR seems to also actively repress translation of specific transcripts which has not been demonstrated before neither in plants nor other eucaryotes. However, this observation fits well the perception of TOR as a promoter of anabolism and repressor of catabolic processes. As already mentioned, TOR promotes translation by phosphorylation of 4E-BP and via phosphorylation of S6K and other mechanisms while it has been shown to repress autophagy by phosphorylation of autophagosome components. So far, inhibition of autophagosome formation by direct phosphorylation of ATGs is the only reported mechanism by which TOR inhibits catabolic turnover. Hence, the underlying results extend our current understanding of how TOR balances anabolism versus catabolism, namely by translational repression of catabolic enzymes. Currently, I can only speculate about the underlying mechanistic details mediating TOR dependent translational repression of CKX enzymes. However, the fact that the translational myc-CKX1 fusion construct does not include the endogenous 5'-UTR, points towards a mechanism that is independent of 5'-UTRs. This is corroborated by the generally very short 5'-UTRs of all CKX isoforms except for CKX4. It can be hypothesized that other translational regulators like eIF2alpha or eIFiso4G could fuel translation of CKXs when TOR is not active. Another possibility would be microRNA mediated translational repression and I have found first hints for a microRNA almost fully complementary to *CKX6* and with a few mismatches also *CKX1* (data not shown). However, this is a very preliminary finding and needs to be tested in future work which has the potential to identify novel pathways mediating translational adaptation in response to environmental change.

TOR regulates root sensitivity towards CK

In contrast to shoots, CK signaling was enhanced in roots after TOR inhibitor even though all relevant CK species were depleted, indicating that root sensitivity for CK was increased. This was corroborated by enhanced expression of all AHK CK receptors, three AHP phosphotransferases and two type-B ARRs. Interestingly, protein levels of the negative regulator AHP6 were strongly reduced while neither the RNAseq data nor a transcriptional pAHP6 reporter pointed towards transcriptional inhibition. This suggested that AHP6 might be translationally repressed upon TOR inhibition and fits well with a potential TOR dependent uORF found in the 5'-UTR leader sequence. Opposite to the shoot, CK functions as negative

regulator of root growth and it is a consistent mechanistic framework if TOR promoted root growth by repression of CK signaling. However, this framework requires further investigation to be confirmed. Root growth assays with AHK, AHP and type-B ARR mutants might render root growth less sensitive towards TOR inhibition similar to CKX mutants in my shoot growth assay. Also monitoring the expression of all CK signaling in a cell type specific manner either with reporter lines or single cell transcriptomics will reveal which cell population contributes most significantly. With this regard, the presumable translational repression of AHP6 is very interesting due to its very stereotypic expression patter in protoxylem (Mähönen et al., 2006). Since TOR inhibition causes increased CK signaling throughout the whole root vasculature it is questionable if translational repression contributes to this phenomenon, especially because *ahp6* mutants do not have obvious growth defects (Mähönen et al., 2006). However, *ahp6* mutants are disturbed in cellular patterning during protoxylem formation and it would be interesting whether TOR inhibition also causes similar patterning defects. Notably, *ipt2/ipt9* double mutants show similar patterning defects in vascular development as *ahp6* mutants (Ko, 2014), which is specifically intriguing as IPT2 and IPT9 mediate isopentylation of tRNAs which are the primary source for *c*Z type CKs. Thus, the observed accumulation of *c*Z species in the root might contribute to TOR mediated growth repression. Even though *c*Zs possess low affinities towards CK receptors they were shown to partially complement vascular patterning defects (Ko, 2014) and since receptor abundance might be increased after TOR inhibition *c*Z residual receptor affinity might be sufficient to enhance CK signaling.

CK as upstream regulator of TOR

After other phytohormones have been shown to serve as upstream regulators of TOR my results now demonstrate that also CK stimulates TOR activity (Li et al., 2017; Schepetilnikov et al., 2017; Wang et al., 2018). Similar as shown for auxin, CK mediated TOR activation requires ROP2, although residual activation was observed in the *rop2-1* mutant background which can be explained by redundancy with ROP6 and ROP4 as shown for auxin (Li et al., 2017; Schepetilnikov et al., 2017). This observation has broad implications regarding CK function. The comparison of my TOR inhibitor transcriptome with a CK meta transcriptome revealed that one third of both datasets (~1500 genes) are commonly regulated by CK and TOR. The common genes were mostly related to translation and ribosome biogenesis which agrees with a recently published proteome study that revealed many ribosomal proteins to be deregulated upon INCYDE or *t*Z treatment (Berková et al., 2020). Together, this indicates that a central function of CK is to activate translation and that TOR contributes to this. In retrospect, this fits well with the fact that CK has been discovered as an agent that stimulates

cell proliferation, a process that requires high levels of translation. Moreover, the observation that CK acts upstream and downstream of TOR fuels a controversy about cell type specific functions of TOR. There are several plausible explanations that arise from these observations. CK and TOR could mutually interfere with each other the same way in all cell types. TOR could regulate CK abundance in one cell type, for example in source tissues for sugars or nitrate, and CK could stimulate TOR activity in other cell types, as for example in sink tissues like the SAM. These scenarios might apply for source and sink tissues simultaneously or exclusively for a source or a sink tissue. Nitrate for example is known to stimulate *tZ* biosynthesis in roots which is then transported to shoots where it fuels development. As TOR has recently been shown to be activated by nitrate one scenario is that TOR is activated in the root where it controls *tZ* abundance, whereupon *tZ* is transported to the SAM stimulating *WUS* expression and shoot growth (Liu et al., 2021). In this scenario TOR would be only required in the root. However, TOR could also control *tZ* abundance locally at the SAM which is not unlikely considering that CKX1, CKX3 and CKX5 are all expressed in the SAM (Bartrina et al., 2011; Werner et al., 2003). At the same time, it is not clear whether TOR is required in the SAM to control WUS expression, particularly because it was shown earlier that signaling initiated by phyB has to come from outside of the SAM to activate *WUS* (Pfeiffer et al., 2016). Another study has recently shown that TOR activity is higher in source tissues in adult leaves compared with young leaves that serve as sink which is consistent with that (Brunkard et al., 2020). However, my grafting results support a model where TOR might act in source and sink tissues. If TOR was functional in shoots, impaired TOR signaling in the roots was not detrimental to shoot development. At the same time, functional root derived TOR signaling was able to partially compensate for a lack of shoot derived TOR. Novel approaches including spatial proteomics and spatial metabolomics are required to resolve TOR activity pattern and might unravel distinct metabolic programs active in plant stem cells as shown for other stem cell systems that favor glycolysis over oxidative phosphorylation (Chandel et al., 2016; Döhla et al., 2022; Ito & Suda, 2014; Takubo et al., 2013).

Differential regulation of autophagy in shoot versus roots

Repression of autophagy is referred to as one of the central aspects of TOR function and it was thus surprising to find several ATG genes repressed after AZD8055 in my shoot transcriptome, particularly since AZD8055 is commonly used as an autophagy inducing agent in studies investigating autophagy (Dauphinee et al., 2019). A GFP-ATG8 cleavage assay confirmed that indeed autophagic flux was not increased in shoots while in roots it was as expected. This further confirms the predictability and confidence of my TOR transcriptome

but also suggests that TOR dependent regulation of autophagy might be different than anticipated so far as it questions the current dogma of TOR as general repressor of autophagy. The most common methods utilized to assess the status autophagy in plants are the GFP-ATG8 cleavage assay also presented here, where autophagic flux is assessed based on the turnover of GFP-ATG8 fusion protein and the accumulation of free GFP, but also microscopy-based assays monitoring the amount of autophagic bodies either based on GFP-ATG8 signal or using the MDC dye that stains acidic compartments. The microscopy-based assays are usually performed in roots or hypocotyls of etiolated seedlings because the high autofluorescence present in leaves makes it very difficult to reliably detect and count autophagic bodies. The GFP-ATG8 cleavage assay is usually performed with whole seedlings which disguises differential effects between tissues. If as demonstrated here autophagy is induced in roots but not in shoots, when analyzing whole seedlings, the accumulation of free GFP in roots will override that no turnover occurs in shoots and hence the result of this assay will be biased towards the situation in the root. It is thus possible that so far tissue specific differences might have been overlooked. A possible explanation might be that shoots are photo-autotrophic while roots are heterotrophic tissues. However, a simpler explanation is that the pharmacokinetics of AZD8055 are just different in roots compared with shoots as roots certainly take up the compound much earlier compared with shoots. Thus, the root response likely precedes the shoot response, and it is possible that at a later time point autophagy will also be induced in shoots. Thorough time course experiments will resolve whether there is a different wiring between TOR and autophagy in autotrophic- versus heterotrophic plant tissues or whether this is simply an artefact of different pharmacokinetics of TOR inhibitors. Nonetheless, my findings stress that it might be important to consider potential tissue specific differences in autophagy dynamics but also to thoroughly characterize temporal and tissue specific dynamics of chemicals used to induce autophagy.

Conclusion

The underlying study exemplarily shows how developmental patterning in plants is coupled with central metabolism and cellular homeostasis. The results demonstrate, how the conserved regulator of metabolism, translation and cellular growth TOR regulates expression of the stem cell master regulator *WUS* by translational repression of CKX catabolic enzymes. In addition, the contribution of CK to TOR mediated control of shoot and root growth is addressed. The underlying mechanism of translational repression of growth factor catabolism extends the known action spectrum of TOR as repressor of catabolism. The derived conceptual framework sheds light on how plants swiftly adapt their growth factor regime in response to

changes in the environment. Due to the ecological benefit that this concept might provide, it is likely that analogous mechanisms apply to a broader range of cellular processes and potentially also exist in other kingdoms of life. Thus, this work builds the basis to identify new regulatory pathways that mediate interactions between organisms and the environment and in addition enables the characterization of novel translational mechanisms that involve TOR as translational repressor.

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Appendix

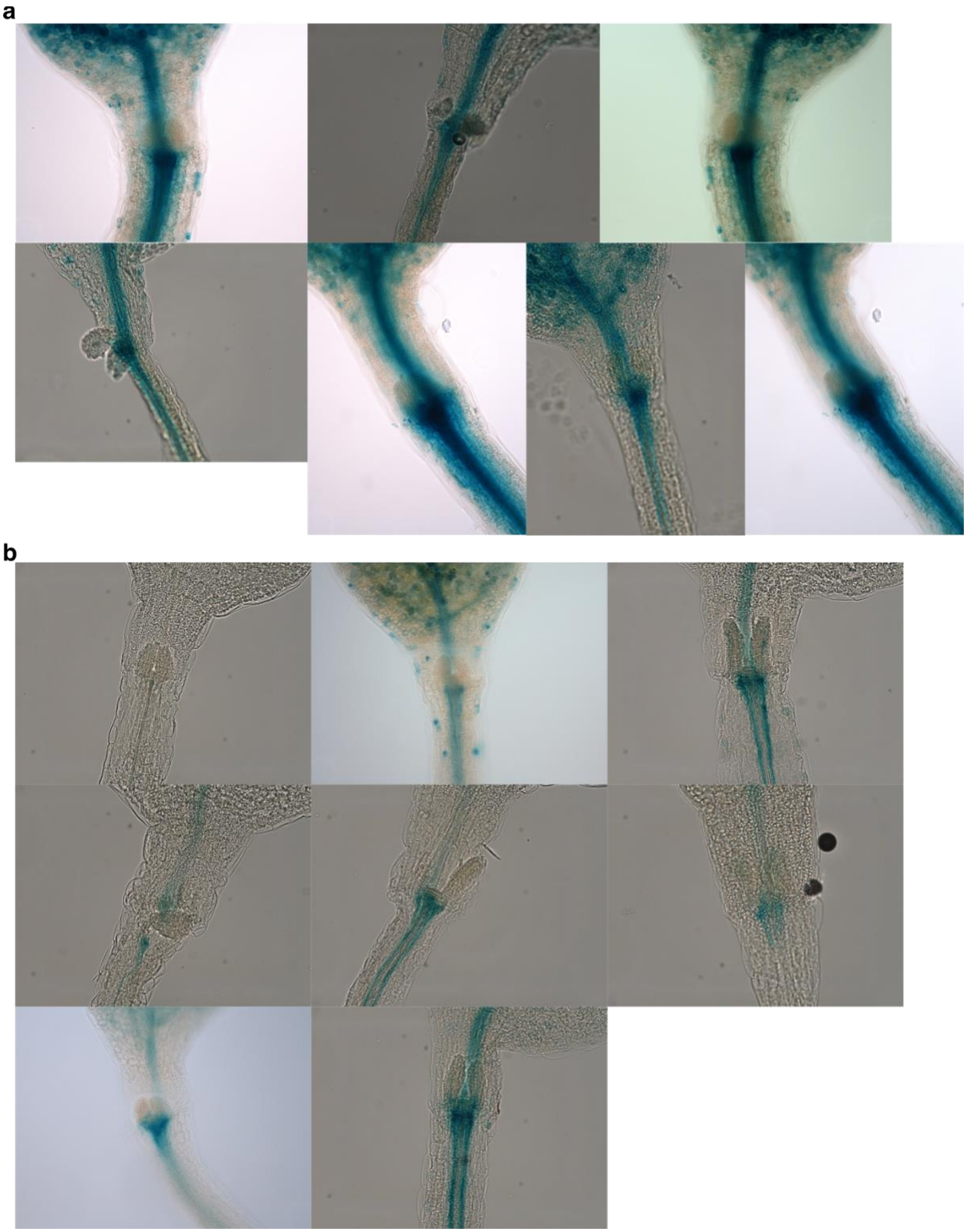

Figure S1: Cytokinin signaling in the SAM is reduced upon TOR inhibition a)+b) Microscopic images of pTCSn:GUS reporter treated for 24 h with a) Mock or b) 2µ AZD8055. Single seedlings of Figure 4c.

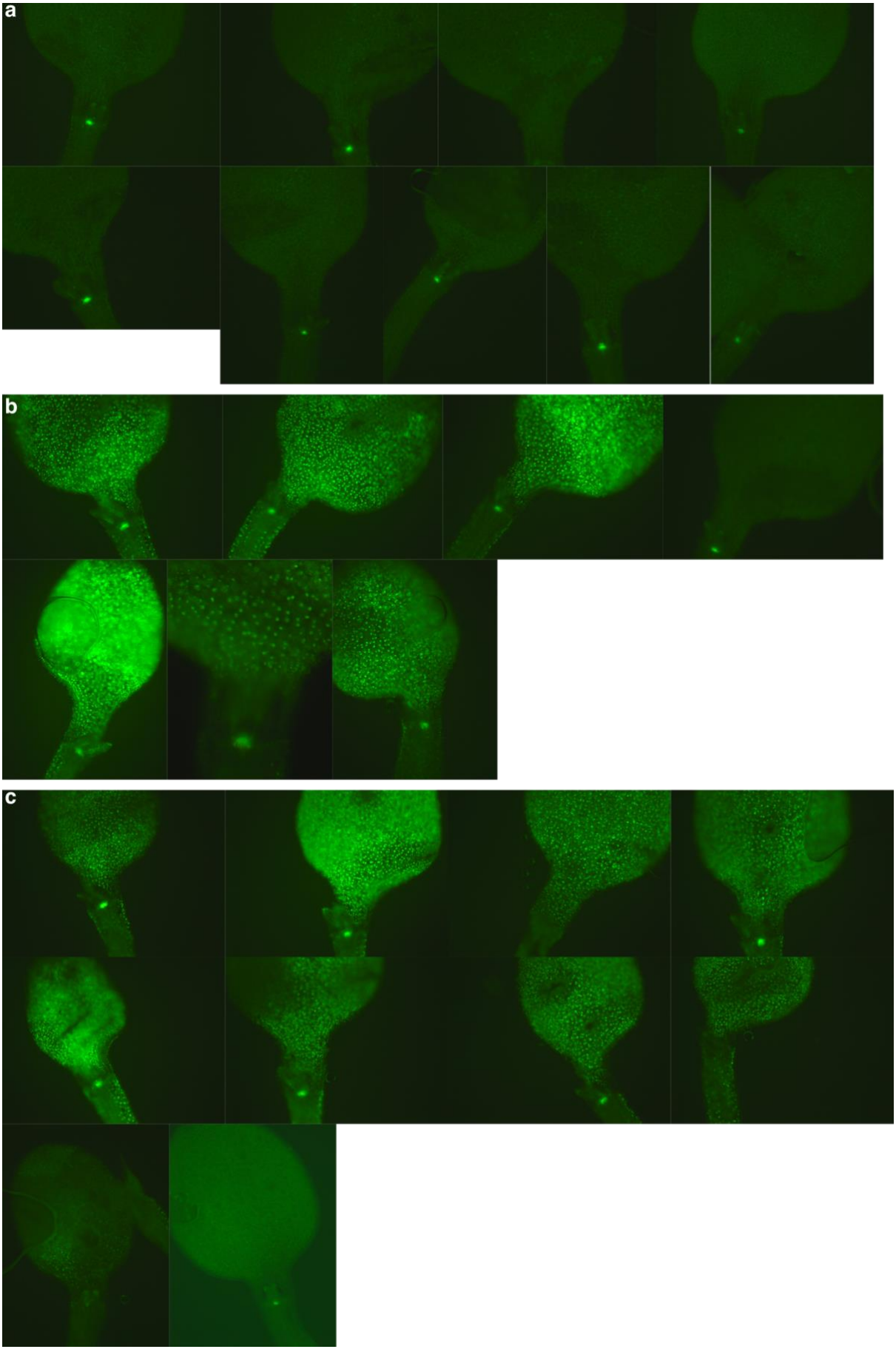
a
b
c

Figure S2: TOR does not affect ARR1∂DDK activity a)-c) Microscopic images of pWUS:3xVenus-NLS reporter line crossed with p35S:ARR1∂DDK-GR. 4 day old seedlings treated with a) mock, b)10 µM dexamethasone (DEX) or c) DEX + 2µM AZD8055. Refers to Figure 6b.

	Genotype	.y.	group1	group2	n1	n2	p	p.signif	p.adj	p.adj.signif
1	ckx5	Fresh.weight	ckx5 0	ckx5 0.1	13	11	0.993	ns	1	ns
2	ckx5	Fresh.weight	ckx5 0	ckx5 0.5	13	12	0.673	ns	1	ns
3	ckx5	Fresh.weight	ckx5 0.1	ckx5 0.5	11	12	0.679	ns	1	ns
4	ckx5	Fresh.weight	ckx5 0	ckx5 1	13	13	8.74E-07	****	8.74E-06	****
5	ckx5	Fresh.weight	ckx5 0.1	ckx5 1	11	13	2.01E-06	****	2.01E-05	****
6	ckx5	Fresh.weight	ckx5 0.5	ckx5 1	12	13	6.15E-06	****	6.15E-05	****
7	ckx5	Fresh.weight	ckx5 0	ckx5 2	13	12	1.74E-09	****	1.74E-08	****
8	ckx5	Fresh.weight	ckx5 0.1	ckx5 2	11	12	5.16E-09	****	5.16E-08	****
9	ckx5	Fresh.weight	ckx5 0.5	ckx5 2	12	12	1.44E-08	****	1.44E-07	****
10	ckx5	Fresh.weight	ckx5 1	ckx5 2	13	12	0.0834	ns	0.834	ns
11	ckx5,6	Fresh.weight	ckx5,6 0	ckx5,6 0.1	12	12	0.799	ns	1	ns
12	ckx5,6	Fresh.weight	ckx5,6 0	ckx5,6 0.5	12	12	0.000475	***	0.00475	**
13	ckx5,6	Fresh.weight	ckx5,6 0.1	ckx5,6 0.5	12	12	0.000211	***	0.00211	**
14	ckx5,6	Fresh.weight	ckx5,6 0	ckx5,6 1	12	11	1.82E-05	****	0.000182	***
15	ckx5,6	Fresh.weight	ckx5,6 0.1	ckx5,6 1	12	11	7.61E-06	****	7.61E-05	****
16	ckx5,6	Fresh.weight	ckx5,6 0.5	ckx5,6 1	12	11	0.29	ns	1	ns
17	ckx5,6	Fresh.weight	ckx5,6 0	ckx5,6 2	12	11	3.23E-08	****	3.23E-07	****
18	ckx5,6	Fresh.weight	ckx5,6 0.1	ckx5,6 2	12	11	1.28E-08	****	1.28E-07	****
19	ckx5,6	Fresh.weight	ckx5,6 0.5	ckx5,6 2	12	11	0.00664	**	0.0664	ns
20	ckx5,6	Fresh.weight	ckx5,6 1	ckx5,6 2	11	11	0.0912	ns	0.912	ns
21	ckx6	Fresh.weight	ckx6 0	ckx6 0.1	12	12	0.592	ns	1	ns
22	ckx6	Fresh.weight	ckx6 0	ckx6 0.5	12	11	0.25	ns	1	ns
23	ckx6	Fresh.weight	ckx6 0.1	ckx6 0.5	12	11	0.0971	ns	0.971	ns
24	ckx6	Fresh.weight	ckx6 0	ckx6 1	12	12	1.19E-05	****	0.000119	***
25	ckx6	Fresh.weight	ckx6 0.1	ckx6 1	12	12	1.75E-06	****	1.75E-05	****
26	ckx6	Fresh.weight	ckx6 0.5	ckx6 1	11	12	0.000791	***	0.00791	**
27	ckx6	Fresh.weight	ckx6 0	ckx6 2	12	12	6.6E-08	****	6.6E-07	****
28	ckx6	Fresh.weight	ckx6 0.1	ckx6 2	12	12	8.87E-09	****	8.87E-08	****
29	ckx6	Fresh.weight	ckx6 0.5	ckx6 2	11	12	7.46E-06	****	7.46E-05	****
30	ckx6	Fresh.weight	ckx6 1	ckx6 2	12	12	0.158	ns	1	ns
31	DR	Fresh.weight	DR 0	DR 0.1	10	12	0.159	ns	1	ns
32	DR	Fresh.weight	DR 0	DR 0.5	10	14	3.29E-05	****	0.000329	***
33	DR	Fresh.weight	DR 0.1	DR 0.5	12	14	0.00235	**	0.0235	*
34	DR	Fresh.weight	DR 0	DR 1	10	15	6.72E-09	****	6.72E-08	****
35	DR	Fresh.weight	DR 0.1	DR 1	12	15	6.97E-07	****	6.97E-06	****
36	DR	Fresh.weight	DR 0.5	DR 1	14	15	0.0185	*	0.185	ns
37	DR	Fresh.weight	DR 0	DR 2	10	13	5.83E-11	****	5.83E-10	****
38	DR	Fresh.weight	DR 0.1	DR 2	12	13	4.61E-09	****	4.61E-08	****
39	DR	Fresh.weight	DR 0.5	DR 2	14	13	0.000261	***	0.00261	**
40	DR	Fresh.weight	DR 1	DR 2	15	13	0.121	ns	1	ns

	Treatment	.y.	group1	group2	n1	n2	p	p.signif	p.adj	p.adj.signif
1	incyde	Freshweight	incyde 0	incyde 0.1	4	5	0.691	ns	1	ns
2	incyde	Freshweight	incyde 0	incyde 0.5	4	4	0.543	ns	1	ns
3	incyde	Freshweight	incyde 0.1	incyde 0.5	5	4	0.305	ns	1	ns
4	incyde	Freshweight	incyde 0	incyde 1	4	4	7.29E-06	****	7.29E-05	****
5	incyde	Freshweight	incyde 0.1	incyde 1	5	4	2.19E-06	****	2.19E-05	****
6	incyde	Freshweight	incyde 0.5	incyde 1	4	4	2.03E-05	****	0.000203	***
7	incyde	Freshweight	incyde 0	incyde 2	4	2	2.25E-08	****	2.25E-07	****
8	incyde	Freshweight	incyde 0.1	incyde 2	5	2	1.02E-08	****	1.02E-07	****
9	incyde	Freshweight	incyde 0.5	incyde 2	4	2	4.05E-08	****	4.05E-07	****
10	incyde	Freshweight	incyde 1	incyde 2	4	2	6.95E-05	****	0.000695	***
11	mock	Freshweight	mock 0	mock 0.1	4	4	0.0553	ns	0.553	ns
12	mock	Freshweight	mock 0	mock 0.5	4	5	0.000667	***	0.00667	**
13	mock	Freshweight	mock 0.1	mock 0.5	4	5	0.0499	*	0.499	ns
14	mock	Freshweight	mock 0	mock 1	4	4	1.82E-06	****	1.82E-05	****
15	mock	Freshweight	mock 0.1	mock 1	4	4	5.35E-05	****	0.000535	***
16	mock	Freshweight	mock 0.5	mock 1	5	4	0.00168	**	0.0168	*
17	mock	Freshweight	mock 0	mock 2	4	2	1.15E-07	****	1.15E-06	****
18	mock	Freshweight	mock 0.1	mock 2	4	2	1.14E-06	****	1.14E-05	****
19	mock	Freshweight	mock 0.5	mock 2	5	2	1.03E-05	****	0.000103	***
20	mock	Freshweight	mock 1	mock 2	4	2	0.00384	**	0.0384	*

Figure S3: Pairwise comparisons of dose response data Table shows pairwise comparisons between different concentrations for each genotype. Values have been calculated using pairwise t-test with Bonferroni correction. Highlighted rows show the concentration where wt (here double reporter (DR)) and *ckx* mutants show altered responses. Refers to Fig 10a.

Figure S4: Pairwise comparisons of dose response data Table shows pairwise comparisons between different concentrations for mock and INCYDE (75 nM) treated seedlings. Values have been calculated using pairwise t-test with Bonferroni correction. Highlighted rows show the concentration where mock and INCYDE treated seedlings show altered responses. Refers to Fig 10c.

Figure S5: Pairwise comparisons of dose response data Table shows pairwise comparisons between different concentrations for each genotype. Values have been calculated using pairwise t-test with Bonferroni correction. Highlighted rows show the concentration where wt and *ckx* mutants show altered responses. Refers to Fig. 10e.

	Genotype	.y.	group1	group2	n1	n2	p	p.signif	p.adj	p.adj.signif
1	ckx2	Fresh.weight	ckx20	ckx20.1	7	7	0.972	ns	1	ns
2	ckx2	Fresh.weight	ckx20	ckx20.5	7	7	0.00342	**	0.0342	*
3	ckx2	Fresh.weight	ckx20.1	ckx20.5	7	7	0.00312	**	0.0312	*
4	ckx2	Fresh.weight	ckx20	ckx21	7	7	2.62E-06	****	2.62E-05	****
5	ckx2	Fresh.weight	ckx20.1	ckx21	7	7	2.37E-06	****	2.37E-05	****
6	ckx2	Fresh.weight	ckx20.5	ckx21	7	7	0.0145	*	0.145	ns
7	ckx2	Fresh.weight	ckx20	ckx22	7	7	1.95E-08	****	1.95E-07	****
8	ckx2	Fresh.weight	ckx20.1	ckx22	7	7	1.78E-08	****	1.78E-07	****
9	ckx2	Fresh.weight	ckx20.5	ckx22	7	7	0.000131	***	0.00131	**
10	ckx2	Fresh.weight	ckx21	ckx22	7	7	0.0833	ns	0.833	ns
11	ckx3	Fresh.weight	ckx30	ckx30.1	7	8	0.877	ns	1	ns
12	ckx3	Fresh.weight	ckx30	ckx30.5	7	7	0.0779	ns	0.779	ns
13	ckx3	Fresh.weight	ckx30.1	ckx30.5	8	7	0.0942	ns	0.942	ns
14	ckx3	Fresh.weight	ckx30	ckx31	7	8	0.000465	***	0.00465	**
15	ckx3	Fresh.weight	ckx30.1	ckx31	8	8	0.000499	***	0.00499	**
16	ckx3	Fresh.weight	ckx30.5	ckx31	7	8	0.0521	ns	0.521	ns
17	ckx3	Fresh.weight	ckx30	ckx32	7	7	9.88E-07	****	9.88E-06	****
18	ckx3	Fresh.weight	ckx30.1	ckx32	8	7	8.76E-07	****	8.76E-06	****
19	ckx3	Fresh.weight	ckx30.5	ckx32	7	7	0.000193	***	0.00193	**
20	ckx3	Fresh.weight	ckx31	ckx32	8	7	0.0262	*	0.262	ns
21	ckx4	Fresh.weight	ckx40	ckx40.1	7	7	0.0487	*	0.487	ns
22	ckx4	Fresh.weight	ckx40	ckx40.5	7	7	0.136	ns	1	ns
23	ckx4	Fresh.weight	ckx40.1	ckx40.5	7	7	0.00119	**	0.0119	*
24	ckx4	Fresh.weight	ckx40	ckx41	7	6	1.55E-05	****	0.000155	***
25	ckx4	Fresh.weight	ckx40.1	ckx41	7	6	7.13E-08	****	7.13E-07	****
26	ckx4	Fresh.weight	ckx40.5	ckx41	7	6	0.000893	***	0.00893	**
27	ckx4	Fresh.weight	ckx40	ckx42	7	7	6.98E-08	****	6.98E-07	****
28	ckx4	Fresh.weight	ckx40.1	ckx42	7	7	4.04E-10	****	4.04E-09	****
29	ckx4	Fresh.weight	ckx40.5	ckx42	7	7	4.46E-06	****	4.46E-05	****
30	ckx4	Fresh.weight	ckx41	ckx42	6	7	0.0991	ns	0.991	ns
31	WT	Fresh.weight	WT0	WT0.1	7	7	0.445	ns	1	ns
32	WT	Fresh.weight	WT0	WT0.5	7	6	3.37E-05	****	0.000337	***
33	WT	Fresh.weight	WT0.1	WT0.5	7	6	0.000264	***	0.00264	**
34	WT	Fresh.weight	WT0	WT1	7	7	6.28E-07	****	6.28E-06	****
35	WT	Fresh.weight	WT0.1	WT1	7	7	5.27E-06	****	5.27E-05	****
36	WT	Fresh.weight	WT0.5	WT1	6	7	0.242	ns	1	ns
37	WT	Fresh.weight	WT0	WT2	7	7	1.12E-08	****	1.12E-07	****
38	WT	Fresh.weight	WT0.1	WT2	7	7	8.33E-08	****	8.33E-07	****
39	WT	Fresh.weight	WT0.5	WT2	6	7	0.0125	*	0.125	ns
40	WT	Fresh.weight	WT1	WT2	7	7	0.137	ns	1	ns

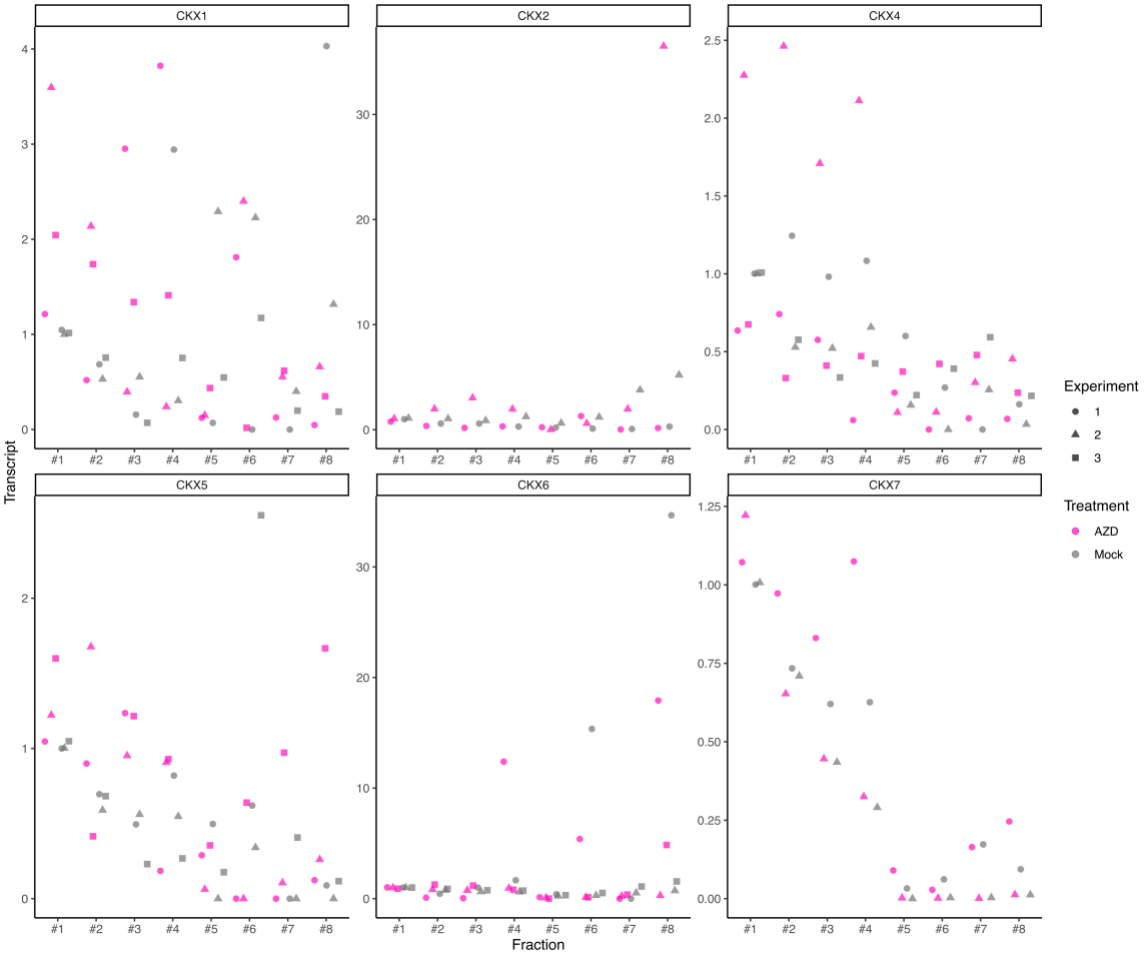

Figure S6: TOR dependent translational regulation of CKX isoforms Ribosome fractionation experiments. Heavy polysomal fractions (fractions 1-3), light polysomal fractions (fractions 4+5) and monosomal fractions(fractions 6-8).CKX transcripts were detected with q-RT-PCR relative to *UBI10* transcript and normalized to the respective mock of fraction #1. Datapoints correspond to independent experimental repetitions corresponding to Figure 13.

Table S3: Primers used for CKX transcript quantification.

ID	Primer Name	Sequence
A08633	CKX7fwd_qPCR	CGGAGTCAATGGTCCAATGC
A08634	CKX7rev_qPCR	GAACCGAAGCAATGCCACAA
A08635	CKX6fwd_qPCR	CCCAGTCATCGTCTACCCAG
A08636	CKX6rev_qPCR	CGATGTTAGGATCGCCACCA
A08637	CKX5fwd_qPCR	GTTCCAACGGCTCTGTTTTGT
A08638	CKX5rev_qPCR	CCGTTGTAAAGACCGATGTCG
A08639	CKX4fwd_qPCR	ATAACGAGGGCCAGGATTGC
A08640	CKX4rev_qPCR	AGTCAACTCCGAGATCATTGGT
A08641	CKX3fwd_qPCR	ACCGCGAAGAAAAGATCCGA
A08642	CKX3rev_qPCR	AACGGCGGAATTAGTGGACA
A08643	CKX2fwd_qPCR	CTCTGGTATCATCGCCGACA
A08644	CKX2rev_qPCR	CTTCGGGACTCGCTCTTCTC
A08645	CKX1fwd_qPCR	TTCCACACAGGCAAGCAGAT
A08646	CKX1rev_qPCR	ACTTGCCAGTTTCCTGATCCAT
A09146	mycCKX1 fwd	AGACTTGAACGGACTCGACG
A09179	mycCKX1 rev	CGAGGAAAGTCTTGTTGTTT
A01067	PP2A_fwd	TAACGTGGCCAAAATGATGC
A01068	PP2A_rev	GTTCTCCACAACCGCTTGGT

Primers used for type-A ARRs were described in (Zhao et al., 2010). Primers used for normalization of polysome data AT4G05320 UBQ10

fwd: GGCCTTGTATAATCCCTGATGAATAAG,

rev: AAAGAGATAACAGGAACGGAAACATAGT

Supplemental information (uploaded on the attached CD)

Supplemental tables S1 and S2 containing DESeq2 result files of RNAseq and the full list of differentially expressed genes. RNAseq raw data and count files is made available as GSE197099 entry at GEO expression omnibus online repository.