TITLE: TARGET OF RAPAMYCIN is essential for asexual vegetative reproduction in Kalanchoë

SHORT TITLE: Kalanchoë asexual reproduction requires TOR Kinase

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AUTHOR CONTRIBUTIONS

M.K. and V.S. designed the project. V.S., K.M., F.J-B., J.B., I.M.V.S. and Z.R. conducted experiments. V.S. and K.M. generated transgenic lines, which were verified and analysed by K.M.. V.S. produced the phylogenetic tree. Z.R. performed Torin2 whole plantlet treatment experiments, whilst K.M. performed leaf margin Torin2 treatments, and J.B. and I.M.V.S. performed AZD-8055 treatment experiments. F.J-B., J.B. and I.M.V.S. performed RT-qPCR. F.J-B. performed pTOR::GUS treatments and staining, and some phenotypic analyses of the transgenic lines. V.S. and K.M. drafted and M.K. revised the manuscript.

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ONE SENTENCE SUMMARY: The highly conserved eukaryotic nutrition-sensing regulator Target Of Rapamycin (TOR) regulates a unique method of asexual reproduction in plants.

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The unique mechanism by which leaf margin cells regain potency and then form a plantlet in *Kalanchoë* spp. remains elusive but involves organogenesis and embryogenesis in response to age, day length, nutrient availability and drought stress. In light of this, we investigated whether TARGET OF RAPAMYCIN (TOR), a conserved protein kinase in eukaryotes that controls cell growth and metabolism in response to nutrient and energy availability, may regulate plantlet formation. *KdTOR* was expressed in the leaf margin at the site of plantlet initiation, in the early plantlet cotyledons, and in the root tip of the developed plantlet. Both chemical and genetic inhibition of TOR Kinase activity in *Kalanchoë daigremontiana* leaves disrupted plantlet formation. Furthermore, downregulation of *KdTOR* in transgenic plants led to wide-ranging transcriptional changes, including decreased *K. daigremontiana* SHOOTMERISTEMLESS and *K. daigremontiana LEAFYCOTYLEDON1* expression, whereas auxin treatments induced *KdTOR* expression in the plantlet roots. These results suggest that the *KdTOR* pathway controls plantlet development in cooperation with auxin, organogenesis, and embryogenesis pathways. The ancient and highly conserved TOR Kinase therefore controls diverse and unique developmental pathways, such as asexual reproduction within the land plant lineage.
INTRODUCTION

Cell differentiation in multicellular organisms confers specialised functions to different tissue types. However, some plants have evolved the ability to reverse this process to regain totipotency. In several Kalanchoë spp. (Crassulaceae), mature leaf cells in the serrations along the leaf margins become totipotent, and form small clonal individuals (plantlets), which detach to form an individual plant (Batygina et al., 1996). Within the Kalanchoë genus, the triggers for this process vary, perhaps in response to the ecological contexts in which they evolved. Some species are unable to produce plantlets (e.g., K. thrysiflora; Fig. 1A); others produce plantlets upon stress induction (e.g., K. pinnata; Fig. 1B-C); while some species produce plantlets constitutively in favourable conditions (e.g., K. daigremontiana; Fig. 1D-E) (Garcês et al., 2007). Phylogenetic analyses suggest that lack of plantlets is the ancestral state, whereas inducible and constitutive plantlet formation are more derived (Garcês et al., 2007).

Most studies to unravel the molecular mechanisms behind plantlet formation have been performed with K. daigremontiana (Garcês et al., 2007, 2014; Liu et al., 2016; Zhu et al., 2017). Whilst a K. daigremontiana plant is capable of forming plantlets throughout its lifespan, the timing of this event is influenced by the age of the plant, the maturity of the leaves, and the environmental conditions such as day length and water availability (Liu et al., 2016). One potential mechanism for this is through the circadian clock gene, SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), the expression of which corresponded with conditions that induced plantlets, such as long days and drought (Liu et al., 2016). Furthermore, overexpression of KdSOC1 reduced plantlet formation, and increased expression of an auxin efflux carrier, PINFORMED1 (PIN1), and auxin content in leaves (Zhu et al., 2017).

Once the appropriate conditions are met, plantlets are produced sequentially from the tip to the base of the leaf, within the serrations in the leaf margin (Johnson 1934). These cells must convert from differentiated mature leaf cells, into stem cells, and organogenesis meristem maintenance genes have been shown to be involved in this process (Garcês et al., 2007). For example, SHOOTMERISTEMLESS (STM) is a KNOTTED1-LIKE HOMEobox (KNOX) gene in Arabidopsis (Arabidopsis thaliana) involved in the maintenance of stem cells in the shoot apical meristem (SAM) (Endrizzi et al., 1996). K. daigremontiana STM (KdSTM) was
expressed in the SAM, the axillary buds, the initial cells which will form the plantlet and the upper part of the cotyledons of a heart-stage plantlet (Garcês et al., 2007). Furthermore, KdSTM RNAi lines prevented plantlet formation in *K. daigremontiana* (Garcês et al., 2007). Consistently, STM expression was absent in leaf margins of *Kalanchoë* species that do not produce plantlets (Garcês et al., 2007).

After plantlet initiation, constitutive plantlet development resembles zygotic embryogenesis, with clear globular and heart stages, and as such has been linked to genetic embryogenesis pathways (Garcês et al., 2007). *A. thaliana* LEAFY COTYLEDON1 (*LEC1*), *LEC2* and *FUSCA3* (*FUS3*) are closely related embryogenesis genes that are expressed during embryo morphogenesis and maturation, and control reserve accumulation and desiccation tolerance of seeds (see review: Braybrook & Harada 2008). Ectopic *LEC1* and *LEC2* expression in Arabidopsis can induce vegetative somatic embryo formation (Lotan et al., 1998; Stone et al., 2001), similar to plantlet formation in *Kalanchoë* (Garcês et al., 2007). In accordance with this, the *KdLEC1* homolog is expressed in the zygotic embryo and the heart-stage of developing plantlets (Garcês et al., 2007). However, *K. daigremontiana* has a truncated LEC1 protein, which cannot rescue the *lec1* mutant when expressed in *A. thaliana*, and is also unable to form viable seeds due to desiccation intolerance (Garcês et al., 2007). This suggests that truncated *LEC1* is required for constitutive plantlet formation, to bypass plantlet dormancy.

Although not fully elucidated, it is clear that *K. daigremontiana* plantlet formation requires complex signalling pathways integrating photoperiod, hormones, embryogenesis and organogenesis. TOR kinase is a highly conserved regulator of cell growth in response to nutrient and energy availability in eukaryotes (see review: Dobrenel et al. 2016), and has been shown to integrate light and hormone availability to control embryogenesis and organogenesis in plants (Menand et al. 2002; Deprost et al. 2005; Moreau et al. 2012; Schepetilnikov et al. 2013; Pfeiffer et al. 2016; Wang et al. 2018). While TOR activity is controlled by the nutrient and energy availability in the cell in all eukaryotes (see review: Dobrenel et al. 2016), the types of nutrients and energy sources in each lineage of eukaryotes vary. For example, TOR in plants is activated by glucose derived from photosynthesis (Xiong et al., 2013). Furthermore, plants independently evolved multicellularity, and have specialised cell types for nutrient acquisition (e.g., leaves and...
roots) as well as their own hormone signalling mechanisms (See Review: Chaiwanon et al., 2016). Of these hormones, the “growth” hormone auxin activates TOR (Schepetilnikov et al., 2013; Li et al., 2017), whilst the “stress” hormone Abscisic Acid (ABA) inhibits TOR (Wang et al., 2018). Furthermore, activation of TOR is tissue specific, as glucose and light are required for TOR activation in the shoot, but only glucose is required in the root (Xiong et al., 2013; Li et al., 2017).

Inducible TOR knockdown lines and chemical TOR inhibition have shown multiple developmental phenotypes, such as smaller leaves, reduced leaf number, delayed lifespan, increased branching and flower sterility (Deprost et al., 2007; Mohammed et al., 2018), and TOR overexpression lines show the opposite phenotypes (Deprost et al., 2007; Ren et al., 2011). Auxin controls many of these processes, and TOR controls the translation of the auxin signalling genes, AUXIN RESPONSE FACTORS (ARFs) (Schepetilnikov et al., 2013). In the presence of glucose but absence of light, exogenous application of the auxin, Indole-3-acetic acid (IAA), activated TOR in the shoot apex (Li et al., 2017). Clearly, auxin is in a complex feedback loop with TOR to control development, by acting both upstream and downstream of TOR function.

TOR kinase also controls expression of organogenesis genes, such as WUSCHEL (WUS) (Pfeiffer et al. 2016). Pfeiffer et al. (2016) found that after 3 days’ incubation with the TOR inhibitor AZD-8055, TOR activity decreased, as did WUS expression. TOR has also been implicated in cellular dedifferentiation during callus formation in Arabidopsis tissue culture. By integrating sugar sensing and E2 PROMOTER-BINDING FACTOR a (E2Fa) phosphorylation, TOR drives transcriptional activation of S-phase genes for cell proliferation to make callus tissues (Lee and Seo, 2017). Furthermore, a study examining metabolic and hormonal profile shifts during in vitro organogenesis in tomato showed that TOR transcripts increased during callus formation (Kumari et al., 2017). Together, these experiments suggest a possible role for TOR in triggering pluripotency in differentiated somatic cells.

Due to TOR’s ability to sense hormone availability and environmental conditions to control embryogenesis and organogenesis, we investigated whether and how Kalanchoë daigremontiana TOR (KdTOR) is involved in plantlet formation. Here we show that KdTOR is expressed during the early stages of plantlet development, and inhibition of its function disrupted plantlet formation, likely through KdSTM and KdLEC1 downregulation.
Furthermore, the plant hormone auxin is also involved in the KdTOR pathway in the plantlet roots. This work reveals the importance of KdTOR as a critical regulator of plantlet formation.

RESULTS

**KdTOR is expressed during plantlet initiation in the leaf margins**

To investigate whether TOR was recruited for plantlet development in Kalanchoë leaves, reporter lines under the control of the KdTOR promoter and 5’ UTR were generated. Three independent lines showing consistent GUS expression patterns were analysed during plantlet development. Within the wild-type leaf indentations, four stages of plantlet development can be distinguished (Fig. 1F-J). Stage 1 is identified by a raised node at the indented region, with no protrusion or visible plantlet (Fig. 1G). Stage 2 is defined by a pedestal, which is a protruding structure that will hold the developing plantlet (Fig. 1H). Next, a visible, pin-shaped plantlet will form from the pedestal (Stage 3; Fig. 1I), before the anisocotylous plantlet cotyledons begin to round (Stage 4; Fig. 1J).

GUS accumulated in the hydathodes of developing leaves (Fig. 2A, arrow) and in the indentations before the onset of plantlet formation (Stage 0; Fig. 2B, arrow). GUS expression was later detected at Stage 1 indentations at the node (Fig. 2C-D, arrows). GUS expression was weak in the pedestal itself (Stage 2; Fig. 2E) but was strongly expressed in the plantlet primordium as it began to emerge (Fig. 2E). The Stage 3 pin-shaped plantlet showed a dramatic reduction in GUS expression (Fig. 2F), compared to the initiating plantlet (Fig. 2E), and it was often undetectable in late stage 3 plantlets (Fig. 2G). Expression across the emerging plantlet was not homogeneous; the tip of the developing cotyledon had less GUS expression than the base (Fig. 2F). No GUS expression was detected in the Stage 4 plantlet cotyledons (Fig. 2H). GUS expression was present in the root primordia of the developing plantlet (Fig. 2I) and was later detected at the root tip of mature plantlets (Fig. 2J). Conversely, no obvious expression was detected in the SAM or leaf primordia of the mature plantlet (Fig. 2K-L), nor in the SAM of the mature plant (Fig. 2M-N).

*KdTOR::GUS* expression suggests that KdTOR is expressed in the early plantlet stages, so to confirm this for native *KdTOR* transcripts, *KdTOR* expression in stages 0-3 was quantified by RT-qPCR. *KdTOR* expression was detected at stages 0-3 of plantlet formation (Fig. 2O). While
RT-qPCR data were not statistically significant, the trend of the expression levels among plantlet developmental stages was comparable to those of pKdTOR::GUS data. This trend of KdTOR expression was repeatedly seen in several independent RT-qPCR experiments. Compared with stage 0, KdTOR expression did not markedly increase during stage 1 of plantlet formation, in agreement with pKdTOR::GUS lines which have similar GUS intensities at these two stages. KdTOR expression in stages 2 and 3 increased compared with stages 0 and 1. RT-qPCR suggests that KdTOR may play a role in plantlet formation throughout the developmental stages, but is most crucial for the initiation of plantlet formation at the pedestal (Stage 2), as also supported by strong pKdTOR::GUS expression.

**Torin2 and AZD-8055 reduced total plant growth and inhibited plantlet formation**

To investigate whether KdTOR has conserved function in controlling growth and development in K. daigremontiana, plantlets were grown in vitro on media containing an ATP-competitive chemical inhibitor that has been shown to inhibit TOR, known as Torin2 (Liu et al., 2013) (Fig. 3A-B). Plant area was significantly lower when grown on 100 µM Torin2 media compared to mock media [P = 0.0013, t = 3.831 (Day 7); P < 0.0001, t = 11.68, 10.36, 8.222 (Day 14, 12, 28)] (Fig. 3A), implying that KdTOR controls plant growth in Kalanchoë. Torin2 treated plants also had significantly fewer leaves on day 7 (P = 0.0419, t = 2.697), 21 (P < 0.0001, t = 12.14) and day 29 (P < 0.0001, t = 10.79; Fig. 3B).

To determine whether KdTOR is involved in plantlet formation, K. daigremontiana leaf margins of plants grown on soil were brushed with 100 µM Torin2 or mock solution. Due to the external method of application, we used a higher Torin2 concentration (100 µM) than previous studies (Montané and Menand, 2013), and the effects of this treatment were confined to plantlet formation without any visible side effects in other parts of the leaf. Firstly, for leaves greater than 3 cm, at all time points the percentage of plants with plantlets was similar when treated with mock or Torin2 solution (Fig. 3C). However, leaves less than 3 cm had a lower percentage of plants with plantlets between day 3 and day 24 when treated with Torin2 compared to mock (Fig. 3D). Overall, Torin2 treated leaves of both sizes had significantly fewer plantlets (In leaves > 3 cm: p = 7.084e-06, F = 20.6754; In leaves < 3 cm: p = 1.159e-14, F = 63.6218; Fig. 3E, F), which was specifically lower from day 12 (P = 0.0404, t = 2.884) through to the final day of measurement for leaves smaller than 3 cm (day 28; P = 0.0012, t = 3.873; Fig. 3F). In younger leaves (less than 3 cm), most indentations are
stage 0 and plantlet formation is yet to initiate, whereas in older leaves (greater than 3 cm), the majority of indentations are stage 1 or 2 and therefore plantlet formation has already been triggered. By the end of the period (29 days after the Torin2 treatment), leaves were completely matured and no additional plantlets were formed, therefore fewer plantlets seen in Torin2 treated leaves were not due to a delay of leaf growth or plantlet initiation.

We further investigated the role of KdTOR in plantlet formation by applying a range of concentrations of ADZ-8055 (2 µM, 20 µM and 40 µM) onto leaves, as ADZ-8055 targets and inhibits TOR more specifically than Torin2 (Chresta et al., 2010; Liu et al., 2013). Overall, AZD-8055 concentrations significantly reduced plantlet formation compared to the mock (In leaves > 3cm: 2 µM p = 0.0005584, F = 12.955; 20 µM p = 0.0397, F = 4.388; 40 µM p = 4.155e-05, F = 19.2924; In leaves < 3 cm: 2 µM p = 0.03633, F = 4.5153; 20 µM p = 0.004928, F = 8.2618; 40 µM p = 0.01193, F = 6.5692; Fig. 3G-J). While higher concentrations broadly showed fewer plantlets (Fig. 3I, J), there was no statistically significant difference among the treatments at different concentrations. No noticeable side effects other than plantlet formation were observed in AZD-8055 treated leaves (Supplemental Fig. S1).

KdTOR silencing lines showed defects in meristem patterning

Whilst informative, chemical inhibition is only transient and may have off target effects (Liu et al., 2013). Therefore 35S::KdTORa silencing lines were generated to investigate how decreased endogenous KdTOR affects plantlet development. For the silencing lines, we amplified and used a 276 bp fragment of KdTOR exon 8 that corresponded to the HEAT repeat domain and showed high conservation across the plant kingdom (Supplemental Fig. S2, Supplemental Table S1). A total of nine independent 35S::KdTORa silencing lines were confirmed by PCR (Fig. 4A, B) and down-regulation of KdTOR expression was confirmed in eight lines (A-H) by RT-qPCR (Fig. 4C) and/or semi-quantitative RT-PCR (Supplemental Fig. S3). All eight lines showed substantial decreases in KdTOR expression compared to wild type (Fig. 4C and Supplemental Fig. S3), suggesting that suppression of KdTOR had been achieved. However, these lines did not show complete down-regulation of KdTOR. This could be explained by the lethality of severe KdTOR knockdowns; only weaker knockdowns survived into adulthood, consistent with embryo lethality in A. thaliana (Menand et al., 2002; Deprost et al., 2007).
Phenotypes varied across lines, but a general reduction in whole plant and leaf size was observed (Fig. 5A, B, C), consistent with TOR repression in other species (Deprost et al., 2007; Xiong et al., 2016; De Vleesschauwer et al., 2018). The most prominent phenotypes observed in these transgenic lines were defects in meristem patterning. Wild-type *K. daigremontiana* forms pairs of leaves in an opposite and decussate phyllotactic order; each pair of leaves is positioned at a 90° angle to the previous pair (Fig. 4D). Two young equally-sized leaf primordia emerge (Fig. 4E) and form a hollow tube-like structure (Fig. 4F), which grows into a pair of young and equally-sized leaves (Fig. 4G). In two independent lines (line D and E), leaves instead emerged three at a time from the main meristem in at least 20% of individuals per line (Fig. 4H-K). After emergence (Fig. 4I), an elongated tube structure consisting of three leaves develops (Fig. 4J), before growth of the equally-sized leaf blades (Fig. 4K). Furthermore, leaves were produced in an alternate phyllotactic order in six independent lines (Fig. 4L). At least 50% of individuals in lines B, C and E, and at least 25% of individuals in lines D, F and G produced leaves alternately, which were unequally sized throughout their development (Fig. 4M-O). In these lines, one leaf emerges at a time (Fig. 4N), instead of a pair seen in wild type (Fig. 4E). These changes to phyllotaxy suggest *KdTOR* may play a role in meristem patterning and leaf initiation in the mature plant, despite not being expressed directly in these tissues (Fig. 2M-N).

*KdTOR* silencing lines have disrupted plantlet formation

All of the seven confirmed transgenic lines that were phenotyped had significantly fewer final plantlet numbers per mature leaf than wild-type plants (*P* < 0.0001; Fig. 5A-D), consistent with chemical leaf margin treatments (Fig. 3E, F, I, J). Mean plantlet number was reduced by at least 85.5% (line E) to as much as 99.3% (line F) compared to wild type (Fig. 5D). In some of the lines with the strongest silencing and most severe phenotypes (*e.g.*, line A and F), plantlet formation was nearly completely abolished (> 99%; Fig. 5B-D). Indentation number was also significantly reduced in transgenic lines relative to wild type (*P* < 0.0001; Fig. 5E-H), by between 51% (line A) and 73% (line G) in all transgenic lines (Fig. 5H). It could therefore be argued that plantlet formation is decreased due to the morphological alteration of the leaf margins in these lines. However, not all reduction of plantlets was due to the loss of indentations. Many normal-looking indentations had no plantlets in these transgenic lines (Fig. 5F, G, K, arrows). Even taking into account the reduction in indentation...
In some indentations, plantlets were not initiated at any stage of the plant's lifespan; notches resembled Stage 0 or terminated following the initial protrusion from the node and subsequent tissue necrosis (Stage 1; Fig. 5K, 6A-B, 6N, 6Q, arrows). At most indentations, however, plantlet formation terminated following the formation of the pedestal (Stage 2), which was often shorter, flattened and lacked the plantlet primordium (Fig. 6C-D, arrow, 6M; 6R). In this case, the termination of plantlet formation in KdTOR silencing lines was not simply delaying plantlet initiation, and it was rather a consequence of defective pedestals incapable of initiating plantlets. In the cases where plantlets were initiated, abnormal plantlet development was observed. Stage 3 plantlets were shorter and thicker, and more exposed on the pedestal, compared to wild type (Fig. 6E-F, S, T). Stage 4 wild-type plantlet cotyledons are anisocotylous (Fig. 6G, O, P, arrows). In some transgenic plants, the large macrocotyledon adopted a bilobed shape early in development (Fig. 6H, arrow). At other stage 4 plantlets, leaves were contorted and bleached (Fig. 6I-J). Mature 35S::KdTORA transgenic plantlet cotyledons were also discoloured and bilobed (Fig. 6K-L). Unlike wild-type plantlets (Fig. 5I), other mature plantlets had smaller, thicker, and misshapen leaves (Fig. 5J), resembling the mother plants (Fig. 5F). Overall, plantlet formation was terminated at each stage across the different lines and KdTOR is therefore likely to be critical for all initial stages of plantlet formation including pedestal development, plantlet primordium initiation and morphological development. These suggested roles of KdTOR are also supported by pKdTORA::GUS expression analyses, in terms of spatio-temporal coincidence.

Expression of key genes for plantlet formation were reduced in KdTOR silencing lines

In order to establish a genetic mechanism by which KdTOR may be regulating plantlet formation in stages 0-3, RT-qPCR was performed in KdTOR silencing lines to measure expression of essential organogenesis (KdSTM) and embryogenesis (KdLEC1) genes. Expression of both KdSTM and KdLEC1 was significantly lower than wild type (Fig. 7A and B),
which suggests that failure to initiate plantlets in some leaf indentations may be due to
downregulation of KdSTM and KdLEC1. Furthermore, expression of leaf crenulation genes
KdJAGGED (KdJAG) and KdCUP-SHAPED COTYLEDON 2 (KdCUC2) decreased in the transgenic
lines (Fig. 7C and D). To confirm wide ranging transcription regulation by TOR, we analysed
genes which have shown to be negatively [RELATED TO AP2 6 (AtRAP2.6L)] and positively
[RIBOSOMAL PROTEIN S5 (ATS5), PROLIFERATING CELLULAR NUCLEAR ANTIGEN 1
(AtPCNA1) and ERBB-3 BINDING PROTEIN 1 (AtEBP)] regulated by TOR activity in published
RNA Seq data sets (Xiong et al., 2013; Dong et al., 2015; Fu et al., 2021). Whilst changes in
KdRAP2.6L were not detectable, we confirmed expected downregulation of KdS5, KdPCNA1
and KdEBP in response to decreased KdTOR expression (Fig. 7E-H).

**Auxin promotes KdTOR expression in the roots**

To investigate whether auxin controls KdTOR expression, as it does TOR activity in
Arabidopsis (Schepetilnikov et al., 2013; Li et al., 2017; Chen et al., 2018), leaves from
pKdTOR::GUS lines generated here were incubated in auxin (IAA) and auxin transport
inhibitor (NPA) solutions. In the hydathode (stage 0), GUS expression was not noticeably
different between mock, 25 µM IAA or 25 µM NPA treatments (Fig. 8A, F, K). Similarly,
exposure to IAA or NPA did not affect expression in the plantlet primordia (stage 2; Fig. 8B,
G, L) or the developing plantlet cotyledons (stage 3; Fig. 8C, H, M). However, plantlet roots
treated with IAA had noticeably higher GUS expression than mock or NPA treated plants
(Fig. 8D-E, I-J, N-O). Whilst GUS expression seemed to be localised to the division zone and
internal cells of the elongation zone in the mock and NPA treated plants, IAA treated roots
also expressed GUS in the epidermis and cortex of the elongation zone. This suggests auxin
promotes KdTOR expression in the root, but not other tissues investigated.

**DISCUSSION**

*K. daigremontiana* plantlet formation is a rare phenomenon in which the ability to regain
potency has been exploited in plantlet development to reproduce asexually by creating
clones (Garcés and Sinha, 2009). In some Kalanchoë species, this method of reproduction is
dependent on the environmental conditions in which the plant grows; plantlet initiation in
*K. daigremontiana* is promoted by long days and drought (Liu et al., 2016). After initiation,
plantlet development involves a combination of organogenesis pathways including KdSTM
(Garcês et al., 2007), and embryogenesis pathways including *KdLEC1* (Garcês et al. 2007; Garcês et al. 2014). Recently, TOR Kinase has emerged as a central player in controlling many aspects of plant development in response to energy and nutrient availability (see review: McCready et al., 2020), as determined in part by environmental conditions. TOR is known to control plant embryogenesis (Menand et al., 2002), promote auxin signalling through *ARF* translation (Schepetilnikov et al., 2013), control meristem function through *WUS* and *YET ANOTHER KINASE 1 (YAK1)* (Pfeiffer et al., 2016; Barrada et al., 2019), and is itself promoted by light, glucose and auxin signalling in the shoot apices (Xiong et al., 2013; Li et al., 2017). Based on their similar reliance on environmental cues and embryogenesis and organogenesis signalling, we investigated the link between TOR kinase and plantlet formation.

Conservation of TOR Kinase amongst eukaryotes highlights its importance in growth and development across the plant and animal kingdoms. TOR phylogenies show protein sequence conservation and therefore possible functional conservation across the land plants and within *Kalanchoë* species (Sapre et al., 2018). Consistent with other eukaryotes, TOR controls cell growth and anabolism in plants (Dobrenel et al., 2016), and all TOR inhibition studies show a reduction in total plant size (Montané and Menand, 2013; Dong et al., 2015; Li et al., 2015, 2017; Xiong et al., 2016). Consistently, growing *K. daigremontiana* plants on the ATP-competitive chemical inhibitor, Torin2, reduced total leaf area. Arabidopsis *tor* knockdown lines also have fewer leaves (Deprost et al. 2007), as *AtTOR* controls leaf initiation by inhibiting cell cycle in response to glucose and light activation (Mohammed et al., 2018). We showed that total leaf number was reduced after growing *K. daigremontiana* plants on Torin2, and so *KdTOR* may similarly be functioning to control *Kalanchoë* leaf initiation.

After establishing that *KdTOR* may have a conserved role in controlling plant size and leaf initiation, the relevance of *KdTOR* in plantlet formation was investigated. Chemical inhibition of *KdTOR* (with both Torin2 and AZD-8055) and *K. daigremontiana KdTOR* knockdown lines displayed a significant reduction in the number of plantlets produced along the leaf margins. This was due to failure to initiate at indentations, as well as plantlet termination at early stages (0-3), rather than delayed initiation, suggesting that *KdTOR* is essential for the initiation and growth of the plantlet from the pedestal. Correspondingly,
PKdT::GUS transgenic lines and RT-qPCR revealed that KDOR was indeed strongly expressed through these early stages, in agreement with A. thaliana AtOR expression in the developing embryo (Menand et al., 2002). Previous RT-PCR studies showed that AtOR mRNA was detectable in many tissue types (Robaglia et al., 2004), whilst pTOR::TOR-GUS line expression was specific to dividing cells (Menand et al., 2002). This suggests post-transcriptional regulation of TOR expression, and therefore our plantlet-specific expression may be due to post-transcriptional regulation of our GUS transcripts, perhaps due to the inclusion of the 5’ UTR in our lines.

Furthermore, KDSTM is an important regulator of stem cell identity and is necessary for inducing plantlet formation in the leaf margins (Garcês et al., 2007). RT-qPCR experiments here suggest that decreased KDSTM expression could be responsible for plantlet disruption in KDOR silencing lines, perhaps through inability to trigger pluripotency in the leaf margins at stages 0-1. Similarly, KdLEC1, which is important for the embryogenesis-like progression of the initiating plantlet (Garcês et al., 2014), also had reduced expression in KDOR silencing lines. These data imply that in favourable conditions, TOR may be promoting KDSTM and KdLEC1 expression for plantlet formation. We therefore provide evidence that KDOR may be acting as the central regulator connecting nutrition sensing with the activation of downstream organogenesis and embryogenesis pathways. KdJAG and KdCUC2 were also downregulated in KDOR antisense lines. JAG and CUC2 are key regulators controlling leaf crenulation (Dinneny et al., 2004; Nikovics et al., 2006), and smooth leaf margins in KDOR lines might suggest KDOR is involved in the leaf crenulation pathway through KdJAG and KdCUC2. Notably, our finding that KDSTM, KdLEC1 and KdJAG expression decreased in KDOR transgenic lines contrasts to the situation in Arabidopsis where TOR does not regulate STM, LEC1 and JAG (Xiong et al., 2013; Dong et al., 2015; Fu et al., 2021). This suggests that KDOR may have acquired unique downstream targets in the leaf to accommodate plantlet formation. We also investigated expression levels of TOR downstream genes KdS5, KdPCNA1, KdEBP and KdRAP2.6L in KDOR plants. In Arabidopsis, inhibition of TOR decreased the expression levels of S5, PCNA1 and EBP but increased RAP2.6L (Xiong et al., 2013; Dong et al., 2015; Fu et al., 2021). Similarly, downregulation of KDOR decreased KdS5, KdPCNA1, KdEBP but did not affect the KdRAP2.6L level. This
suggests that KdTOR is likely to retain the regulatory role in metabolic pathways in the Kalanchoë leaf, similar to that of Arabidopsis.

Alongside a reduction in plantlet number, changes to plantlet morphology were also observed at all developmental stages. The plantlet cotyledons of KdTORa silencing lines were misshapen and white in colour, suggesting a loss of chlorophyll production. This is consistent with previous studies in Arabidopsis, in which TOR activity promoted chlorophyll biosynthesis during cotyledon greening through ABA INSENSITIVE 4 (ABI4) (Li et al., 2015). However, pKdTOR::GUS expression was not detected beyond stage 3 cotyledons. Notably, our data showed that some defective phenotypes in KdTOR transgenic plants manifested in a region where pKdTOR::GUS expression was absent, suggesting that some KdTOR actions may be cell non-autonomous. For example, altered phyllotaxy was observed in the absence of GUS expression in the SAM. This mismatching expression can also be seen in some Arabidopsis phenotypes; expression of an AtTOR::GUS fusion protein was strongest in the A. thaliana embryo, the SAM and flower buds, but was absent from differentiating tissue such as expanding leaves (Menand et al., 2002), in which many tor phenotypes such as reduced leaf size and chlorophyll reduction are observed (Deprost et al., 2007). It will be informative to determine if the KdTOR protein or transcript is mobile, or is controlling indirect downstream signalling in distant tissues, despite the absence of in situ expression in our pKdTOR::GUS lines.

pKdTOR::GUS expression did however occur strongly in the root primordia of the developing plantlet, and was consistently detected at the tip of the growing root. AtTOR expression in root meristems has previously been reported in A. thaliana (Menand et al., 2002), and the recent discovery that YAK1 is negatively regulated by TOR to promote root meristem maintenance (Barrada et al., 2019) provides scope for investigation of TOR as a conserved regulator of root meristem genes. In addition, KdTOR was expressed at the hydathode of the leaf. Similar GUS expression patterns in hydathodes and root tips are present in the auxin signalling reporter DRS::GFP Arabidopsis lines (Bilsborough et al., 2011), indicating that auxin and TOR may be interacting and this auxin localisation is conserved in Kalanchoë. Changes to phyllotaxy in the main meristem of KdTORa lines also support the suggestion that KdTOR may be involved in downstream auxin signalling. Furthermore, we show that auxin promoted KdTOR expression in the plantlet root but not in the hydathode or the...
plantlet primordium. This tissue specific TOR expression is reminiscent of TOR protein activity in Arabidopsis; whilst auxin-Rho of Plants (ROP) signalling activated TOR to trigger S phase in the SAM, auxin was not implicated in the activation of AtTOR-E2Fa signalling to activate the root apical meristem (Xiong et al., 2013; Li et al., 2017). It is important to note that the results presented here show only the changes in KdTOR gene expression, not protein activity, and it is possible that auxin activation of the KdTOR protein may be occurring in the hydathode or the plantlet, as it does in Arabidopsis (Chen et al., 2018). Auxin is also known to activate AtTOR to promote selective translation of ARF genes (Schepetilnikov et al., 2013; Li et al., 2017), so determining if this signalling module is conserved in Kalanchoë may elucidate the auxin-TOR-plantlet signalling pathway.

In conclusion, we have demonstrated that TOR’s conserved role as central mediator of environmental signals and developmental responses extends to the unique process of K. daigremontiana plantlet formation. How directly TOR controls developmental genes remains to be determined, both in K. daigremontiana and A. thaliana. Asexual reproduction in Kalanchoë species represents a unique innovation, requiring the reversion of differentiated cells to a totipotent state and recruitment of organogenesis and embryogenesis regulators. The confirmation that TOR signalling plays a key role in this process demonstrates how a conserved eukaryotic signalling pathway has been adopted for a novel Kalanchoë-specific process. As an ancient and robust signalling mechanism, TOR may have been recruited to integrate myriad environmental and nutritional information to ensure timely plantlet formation under favourable conditions, for optimal asexual reproduction. Due to their sessile nature, plants have evolved incredible resilience to alter their developmental and metabolic pathways in response to nutrient and energy availability. The TOR pathway plays a pivotal role during these developmental events and has contributed to the remarkable diversity within the plant kingdom.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Wild-type (WT) and transgenic Kalanchoë daigremontiana plantlets were potted into a mix of Levington’s F2 compost (Scott’s Miracle Gro, UK), Perlite (Sinclair Horticulture Ltd, UK),
and Vermiculite (Sinclair Horticulture Ltd, UK) in a 6:1:1 ratio and grown in an MLR-350
Versatile Environmental Test Chamber (Sanyo, Japan) in long day conditions (16 hours light,
8 hours dark, 680 LUX) at 23 °C.

**Treatment Conditions**

Mature plantlets were taken from leaves and sterilised for 3 minutes in 20 % (v/v) Sodium
Hypochlorite and 0.001% (v/v) Triton X-100 (Fisher Scientific, UK). One plantlet per well was
grown in 6 well plates on ½ Murashige and Skoog media (Duchefa Biochemie, The
Netherlands) containing either 100 µM Torin2 (Sigma-Aldrich, UK) and 0.5 % (v/v) DMSO
(Sigma Aldrich-UK) or just 0.5 % (v/v) DMSO (mock). Plant area was recorded every week for
four weeks.

To treat *K. daigremontiana* leaves, plantlets were potted and grown for 6 weeks. Torin2
solutions [100 µM Torin2, 0.5 % (v/v) DMSO and 0.5 % (v/v) Tween-20 (AppliChem, USA)] or
AZD-8055 solutions [2 µM, 20 µM or 40 µM AZD-8055 (Selleckchem, USA), 0.5 % (v/v) DMSO
and 0.5 % (v/v) Tween-20 (AppliChem, USA)] and mock solutions (0.5 % (v/v) DMSO and 0.5
% (v/v) Tween-20) were applied by brushing the margins of plastochron 2 (*P*₂) leaves. Leaf
size was recorded before application, and plantlet number was recorded every three days
for 27 days (Torin2 treatment) or every 7 days for 35 days (AZD-8055 treatment). Plantlets
were counted along one margin of one leaf per plant.

To treat *pKdTOR::GUS* lines, leaves of *P*₂ and mature plantlets from three independent lines
were incubated in 3-Indoleacetic acid (IAA) solutions [25 µM IAA (Sigma-Aldrich, UK), 0.1 %
(v/v) DMSO and 0.5 % (v/v) Tween-20], Naphthylphthalamic acid (NPA) solutions [25 µM
NPA (Fluka® Analytical, Switzerland), 0.1 % (v/v) DMSO and 0.5 % (v/v) Tween-20] and mock
solutions (0.1 % (v/v) DMSO and 0.5 % (v/v) Tween-20) for 24 hours. Treated leaves were
then transferred to GUS Staining solution.

**ß-Glucuronidase (GUS) Staining**

*pKdTOR::GUS* leaf margins and mature plantlets were incubated in GUS Staining solution
[100 mM Sodium phosphate Buffer pH 7.2 (BDH Chemicals, UK), 10 mM EDTA pH 8
(Promega, USA), 0.1 % (v/v) Triton X-100 (Fisher Scientific, UK), 1 mM Potassium
ferricyanide (III) (Sigma-Aldrich, UK), 1 mM Potassium ferrocyanide (Sigma-Aldrich, UK), 2
mM X-GlcA (Melford, UK)]. Tissues were incubated in the dark for 24 hours, then cleared in
100 % (v/v) ethanol.

**Gene Cloning and Vector Assembly**

Degenerate primers for *KdTOR* were designed against aligned *K. laxiflora* and *K. fedtschenkoi* sequences (obtained from Phytozome v12.1, JGI, University of California). For *KdTOR* antisense constructs, a 276 bp fragment of *KdTOR* exon 8 was cloned using gene specific primers (Supplemental Table S2). The primers for the *KdTOR* promoter fragment were designed to amplify 1466 bp upstream of the start codon including the entire 5’ UTR region (Supplemental Table S2). The *KdTOR* exon 8 and promoter sequences were cloned using Q5® High Fidelity DNA Polymerase (New England Biolabs, USA) then ligated into pGEM®-T Easy (Promega, USA) after gel extraction (Nucleospin® gel and PCR Clean-Up Kit; Macherey-Nagel, Germany). Using Golden Gate assembly, the *KdTOR* exon 8 fragment was ligated in an antisense orientation with the cauliflower mosaic virus (CaMV) 35S Promoter and Terminator into a modified pBI121 vector (35S::*KdTOR*α). The promoter fragment of *KdTOR* was assembled with the coding region of *GUS* and the *Nopaline Synthase* (*Nos*) Terminator into the modified pBI121 vector (*pKdTOR*:GUS). Ligated constructs were then transformed in *Escherichia coli* strain *DH5α* for selection. Once confirmed, correct constructs were transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation and checked with culture PCR (Supplemental Table S2).

**Kalanchoë daigremontiana** transformation

Wild-type *K. daigremontiana* plants were transformed with 35S::*KdTOR*α or *pKdTOR*:GUS as previously described (Garcês & Sinha 2009).

**Genotyping and Phenotyping transgenic lines**

DNA was extracted according to the ‘Quick DNA prep for PCR’ protocol (Weigel and Glazebrook, 2002). PCR was performed with Q5® High-Fidelity DNA polymerase and BioTaq™ polymerase (Bioline, UK) and *KdTOR*α and 35STerm reverse primers (Supplemental Table S2). Additional PCR checks were carried out with NPTII forward and reverse primers (Supplemental Table S2). Cycling conditions were set according to the Q5® protocol, with annealing temperature of 58 °C and extension for 30 seconds.
Plantlet number and indentation number of each transgenic line were recorded at leaf maturity (~5 weeks). No further plantlets were formed after this time period.

**RNA Extraction and cDNA synthesis**

The indented notches of wild-type *K. daigremontiana* leaves at each plantlet formation stage (Stage 0-3) and P1 leaves of individual 35S::*KdTORa* lines (Stage 0) were excised and frozen in liquid nitrogen. Several notches of the same stage were harvested from different leaves and grouped together into one sample to have enough tissue for RNA extraction. For *KdSTM* and *KdLEC1* RT-qPCR, the margins of newly emerging leaves (P1) were harvested. Total RNA was extracted with the RNeasy Plant Mini Kit (Qiagen, USA), using 10 mg Polyvinylpyrrolidone (PVP, MW=40000) dissolved in 600 µl RLC Buffer per 100 mg ground tissue. RQ1 DNase (Promega, USA) and Tetro cDNA Synthesis (Bioline, UK) kits were used, according to the manufacturers’ protocols. cDNA synthesis reactions proceeded for 1 hour at 45 °C using a mixture of Random Hexamer and Oligo d(T) primers (Bioline, UK).

**Reverse Transcriptase Quantitative PCR (RT-qPCR) and RT-PCR**

For RT-qPCR, a StepOnePlus™ Real-Time PCR machine with StepOne™ Software v2.3 was used, with a SensiFAST™ SYBR Hi ROX Kit (Bioline, UK). *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE* (*KdGAPDH*) and 18S ribosomal RNA (*Kd18S*) were used as control genes (Supplemental Table S2; Garcês et al., 2014) with an annealing temperature of 60 °C. Expression of these genes did not change in RNA Seq data sets Xiong et al., 2013, Dong et al., 2013 and Fu et al., 2021. Three biological replicates and three technical replicates were used; three independent lines were chosen, total RNA was extracted from three plants per line (nine plants in total), and the cDNA of each plant was tested three times for RT-qPCR. *qKdTOR* primers (Supplemental Table S3) were designed in exon 35 and 36 respectively. The Comparative CT method was used for analysis.

For Reverse Transcription (RT)-PCR, 1X NH₄ Reaction Buffer, 1.5 mM MgCl₂, 1 mM dNTPs, 1 mM forward and reverse primers (Supplemental Table S3), 2.5 ng/µl cDNA, 10 µl/ml BioTaq™ Polymerase and 10 µl/ml Q5® High-Fidelity Polymerase were mixed in a final volume of 20 µl. A thermal cycling reaction was run according to settings recommended by the BioTaq™ protocol, with annealing at 58 °C and extension for 30 seconds, for 39 cycles. *GAPDH* was used as a loading control and identical settings were used for 35 cycles.
Image Acquisition and Data Analysis

Photographs of *K. daigremontiana* were taken using a Huawei P smart (FIG-LX1) with an Apexel 10x Macro camera attachment. A GXCAM Eclipse (0654) Wi-Fi camera attached to a S8APO Stereo Microscope (Leica, USA) was used to visualise notches. Fiji Image J (http://imagej.net/Fiji/Downloads) was used to calculate plant areas and add scale bars. All graphs and statistical analyses were produced using GraphPad Prism Version 8.41. Two Way ANOVA (Repeated Measures) with Sidak’s Multiple Comparisons tests (95 % Confidence limits) were performed on the Torin2 treatment data. One Way ANOVA with Dunnett’s Multiple Comparisons tests were performed on the 35S::KdTORa plantlet data.

SEM Analysis

*K. daigremontiana* leaves with plantlets at different developmental stages were fixed for SEM and viewed as described previously (Garcês et al., 2016; Zoulias et al., 2019).

Phylogenetic Tree Construction

After sequencing the 276 bp KdTOR exon 8 fragment, the predicted *K. daigremontiana* peptide sequence (92 amino acids) was aligned with full length TOR peptide sequences from 38 other eukaryotic species. To obtain TOR orthologs for this alignment, a tBLASTn search was performed using the *Arabidopsis thaliana* TOR peptide sequence (NP_175425.2) as a query. Where possible, the Reference RNA Sequences (refseq_rna) database on NCBI BLAST was used with default parameters (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). If species were absent from this list, a tBLASTn search was performed in Phytozome against their genomic databases with default parameters (https://phytozome.jgi.doe.gov/pz/portal.html#search?show=BLAST). Species names, sequence accessions, databases used, and dates accessed can be found in Supplementary Table S1. Peptide sequences were aligned using MUSCLE with default parameters in MEGA X 10.1 software for macOS (Kumar et al., 2018; Stecher et al., 2020). The aligned sequences were trimmed to the 92 amino acid region homologous to the *K. daigremontiana* TOR fragment before performing a maximum likelihood test using all sites to predict the best fit model for phylogenetic analysis. Based on these results, a maximum likelihood tree with a Jones-Taylor-Thornton (JTT) model and Gamma (G) distributed substitution rate was
constructed in MEGA X. The tree was rooted on the *Mus musculus* TOR peptide sequence. Any sequences that fell outside of the plant TOR monophyly were removed as they are unlikely to be TOR homologs. 500 bootstrap replicates were performed.

**ACCESSION NUMBERS**

The 276 bp *KdTOR* exon 8 fragment has the GenBank accession number MT955591.

**SUPPLEMENTAL DATA**

- **Supplemental Figure S1.** Plantlet formation 35 days after AZD-8055 treatments.
- **Supplemental Figure S2.** Alignment and phylogeny of *KdTOR* with divergent plant species.
- **Supplemental Figure S3.** Semi-quantitative RT-PCR in TOR antisense lines.
- **Supplemental Table S1.** Sampling strategy for TOR phylogenetic tree construction.
- **Supplemental Table S2:** List of primers used for gene cloning.
- **Supplemental Table S3:** List of primers used for genotyping and RT-qPCR.

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**FIGURE LEGENDS**

**Figure 1.** Plantlet formation in *Kalanchoë*. A-E Kalanchoë species evolved different strategies in terms of plantlet formation. *Kalanchoë thyrsiflora* does not make plantlets (A), whereas *Kalanchoë pinnata* makes plantlets when leaves are detached (B, C). *Kalanchoë daigremontiana* constitutively makes plantlets along the margins of the leaves in favourable conditions (D and E). F-J, Stages of wild-type *K. daigremontiana* plantlet formation. F, Stage 0: young leaf indentation with no evidence of pedestal or plantlet. G, Stage 1: no pedestal has formed and the margin is visibly raised at the node. H, Stage 2: a pedestal has formed. I, Stage 3: a thin, pin-shaped plantlet emerges from pedestal which is visible with the naked eye. J, Stage 4: plantlet cotyledons begin to elongate and become rounder. Scale bars: 5 cm (A-E); 200 µm (F-J). co; cotyledon, no; node, pe; pedestal, pl; plantlets, pp; plantlet primordium.

**Figure 2.** Expression of *KdTOR* through *Kalanchoë daigremontiana* plantlet formation. A-G, *pKdTOR::GUS* lines showed *GUS* expression through stages 0-3 of plantlet formation. Signal was detected at the hydathode (A, arrow), the indentation of the leaf margin (B, arrow), and at the node within the indentation (C-D, arrows). At the pedestal, *GUS* was expressed in the initiating plantlet primordium (E) and in the early stages of cotyledon development (F), but not in the expanding cotyledons (G-I). Whilst signal was detected in the root primordia (I) and the root tips of mature plantlets (J), GUS did not accumulate in the SAM of plantlets (K-L) or mature plants (M-N). O, RT-qPCR of *KdTOR* expression in Stages 1-3, relative to Stage 0. One-Way ANOVA with Dunnet’s Multiple comparison, (P < 0.05; n=3). Error bars show SEM. Scale bars: 1 mm (A, I, J, K, M); 0.5 mm (L, N); 200 µm (B-H). co; cotyledon, pe; pedestal, pp; plantlet primordium, rp; root primordium, ro; root, lp; leaf primordium, SAM; shoot apical meristem.

**Figure 3.** Torin2, AZD-8055 and mock treatment of *Kalanchoë daigremontiana*. A and B, Area and leaf number of plantlets planted on media containing mock solution or 100 µM Torin2. C-F, 100 µM Torin2 brushed directly onto the leaf margins reduced plantlet formation (n=25). G-J, 2 µM, 20 µM and 40 µM AZD-8055 brushed directly onto the leaf margins reduced plantlet formation independent of leaf size (greater than 3 cm, n=32, less than 3 cm, n=45). Error bars show SEM. Two Way ANOVA (Repeated Measures) with Sidak’s Multiple Comparisons tests (95 % Confidence limits: ns; P > 0.05, *; P ≤ 0.05, **; P ≤ 0.01, ***; P ≤
0.001, ****; P ≤ 0.0001) (A, B, E, F) and Least Squares Mean with Tukey’s P value adjustment (95 % Confidence limits) (I, J).

Figure 4. Genotypic analysis and phyllotaxy phenotypes of 35S::KdTORa silencing lines. A-C, PCR confirms 35S::KdTORa lines are transgenic and KdTOR is downregulated. Amplification of transgene (A) and NPTII (B), and RT-qPCR of KdTOR expression in independent 35S::KdTORa lines (C). Negative control (-): wild type; positive control (+): KdTOR::pBI128 plasmid. Kd18S was used as a control for C. One-Way ANOVA with Dunnett’s Multiple comparison, n=3. ns; P > 0.05, *; P ≤ 0.05, **; P ≤ 0.01, ***; P ≤ 0.001, ****; P ≤ 0.0001. Error bars show SEM. D-O, Whole plant phenotypes in 35S::KdTORa lines. D-G, Wild-type K. daigremontiana leaves emerge in pairs in an opposite and decussate phyllotactic order (D). After the two leaf primordia emerge (L1 and L2, E), a hollow tube-like structure is formed (F), before growth of the two equally sized leaves (G). H-K, 35S::KdTORa leaves can emerge three at a time from the same node (H). Three similarly sized leaves can be detected from emergence to expansion (I-K). L-O, 35S::KdTORa leaves can emerge in an alternate phyllotactic order (L), forming one leaf at a time (M). Consequently, the older leaves (L3, L2) are larger than the younger leaves (L1) (N, O). Scale bars: 1 cm (D, H, L, G, K, O); 400 µm (E, I, M); 1 mm (F, J, N). L1; Leaf 1, L2; Leaf 2, L3; Leaf 3.

Figure 5. Phenotype analysis of plantlets in Kalanchoë daigremontiana 35S::KdTORa silencing lines. A-D, KdTOR silencing significantly reduced plantlet formation. In wild-type K. daigremontiana, plantlets are produced along the leaf margins (A). As illustrated in lines A (B) and H (C), all transgenic lines had significantly fewer plantlets per leaf (P Value <0.0001) than wild type (D). E-H, KdTOR silencing significantly reduced indentation of the leaf margin. Wild-type K. daigremontiana have regular leaf margin indentations where plantlets form (E). Margins of KdTOR silencing lines appeared smooth and irregular (F, G), and had significantly fewer indentations (P value <0.0001) in the leaf margins than wild type (H). I-L, The indentations of KdTOR leaves were rarely occupied by a plantlet. In wild-type plants, nearly all indentations along the leaf margin are occupied by a plantlet (I), whereas transgenic plants have a lower percentage of indentations occupied (L) with a plantlet (J) than without (K). One-Way ANOVA with Dunnett’s Multiple comparison (n = 9). Error bars show SEM. Scale bars: 1 cm.
Figure 6. Defective 35S::KdTORa plantlet development at the indentation. A-L, KdTOR silencing lines display defects in plantlet development at all stages of plantlet formation. The tissue rises at the node in wild type at stage 1 (A), however, in some KdTOR silencing lines, raised tissues at the node were aborted or became necrotic (B). Compared to wild type (C, E), the pedestal was misshapen and not visible (stage 2, D), or plantlet cotyledons emerging from the pedestal were misshapen and exposed on the shortened pedestal (Stage 3, F) in transgenic lines. Whilst the two wild type cotyledons at stage 4 were beginning to round and stayed round (G, I, K), the larger cotyledon of the transgenic plantlet often developed a bilobed shape (H). If the transgenic cotyledons did not become chlorotic and shrivelled (J), they retained their bilobed shape at maturity (L). M-T, Scanning electron microscope images of wild type (M-P) and 35S::KdTORa indentations (Q-T), showing Stage 1 (M, Q), 3 (N, O, R, S) and 4 (P, T) plantlets. In transgenic plants, plantlet formation aborted prior to pedestal formation (Q) or terminated at the pedestal, leaving necrotic tissue (R) or non-viable plantlet structures (S, T). Scale bars: 200 µm (A-J; M-T); 1 cm (K-L).

Figure 7. RT-qPCR analysis of 35S::KdTORa silencing lines. A-D, RT-qPCR of KdSTM, KdLEC1, KdJAG and KdCUC2 expression in 35S::KdTORa silencing lines, relative to wild type (WT). E-H, RT-qPCR of known TOR downstream genes, KdRAP2.6L, KdS5, KdPCNA1 and KdEBP. Kd18S was used as control. One-Way ANOVA with Dunnet’s Multiple comparison, n=3. ns; P > 0.05, *; P ≤ 0.05, **; P ≤ 0.01, ***; P ≤ 0.001, ****; P ≤ 0.0001. Error bars show SEM.

Figure 8. Auxin treatment of pKdTOR::GUS lines to test upstream activation of KdTOR. pKdTOR::GUS leaves were incubated in mock solution (A-E), 25 µM IAA (F-J), or 25 µM NPA (K-O) for 24 hours. There were no differences in expression in the hydathodes (stage 0; A, F, K), in the plantlet primordia (stage 2; B, G, L), or in the developing cotyledons (stage 3; C, H, M). However, roots of plantlets treated with IAA had stronger GUS expression in the epidermis and cortex of the elongation zone (I-J) when compared to mock (D, E) or NPA treated (N, O) roots. Scale bars: 500 µm (A, F, K); 100 µm (B, G, L); 200 µm (C-E, H-J, M-O).

REFERENCES

Barrada, A., Djendli, M., Desnos, T., Mercier, R., Robaglia, C., Montané, M. H. and Menand, B. (2019) ‘A TOR-YAK1 signaling axis controls cell cycle, meristem activity and plant growth in Arabidopsis.’ Development (Cambridge, England), 146(3).
Batygina, T. B., Bragina, E. A. and Titova, G. E. (1996) ‘Morphogenesis of Propagules in Viviparous Species Bryophyllum Daigremontianum and B. Calycinum.’ *Acta Societatis Botanicorum Poloniae*, 65(1–2) pp. 127–133.

Bilsborough, G. D., Runions, A., Barkoulas, M., Jenkins, H. W., Hasson, A., Galinha, C., Laufs, P., Hay, A., Prusinkiewicz, P. and Tsiantis, M. (2011) ‘Model for the regulation of Arabidopsis thaliana leaf margin development.’ *Proceedings of the National Academy of Sciences of the United States of America*, 108(8) pp. 3424–3429.

Braybrook, S. A. and Harada, J. J. (2008) ‘LECs go crazy in embryo development.’ *Trends in Plant Science*, 13(12) pp. 624–630.

Chaiwanon, J., Wang, W., Zhu, J. Y., Oh, E., and Wang, Z. Y. (2016) ‘Information Integration and Communication in Plant Growth Regulation.’ *Cell*. Elsevier Inc., 164(6) pp. 1257–1268.

Chen, G. H., Liu, M. J., Xiong, Y., Sheen, J. and Wu, S. H. (2018) ‘TOR and RPS6 transmit light signals to enhance protein translation in deetiolating Arabidopsis seedlings.’ *Proceedings of the National Academy of Sciences of the United States of America*, 115(50) pp. 12823–12828.

Chresta, C. M., Davies, B. R., Hickson, I., Harding, T., Cosulich, S., Critchlow, S. E., Vincent, J. P., Ellston, R., Jones, D., Sini, P., James, D., Howard, Z., Dudley, P., Hughes, G., Smith, L., Maguire, S., Hummersone, M., Malagu, K., Menear, K., Jenkins, R., Jacobsen, M., Smith, G. C., M., Guichard, S. and Pass, M. (2010) ‘AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity.’ *Cancer Research*, 70(1) pp. 288–298.

Deprost, D., Truong, H. N., Robaglia, C. and Meyer, C. (2005) ‘An Arabidopsis homolog of RAPTOR/KOG1 is essential for early embryo development.’ *Biochemical and Biophysical Research Communications*, 326(4) pp. 844–850.

Deprost, D., Yao, L., Sormani, R., Moreau, M., Leterreux, G., Bedu, M., Robaglia, C. and Meyer, C. (2007) ‘The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation.’ *EMBO Reports*, 8(9) pp. 864–870.

Dinneny, J. R., Yadegari, R., Fischer, R. L., Yanofsky, M. F. and Weigel, D. (2004) ‘The role of
JAGGED in shaping lateral organs. ‘Development, 131(5) pp. 1101–1110.

Dobrenel, T., Caldana, C., Hanson, J., Robaglia, C., Vincentz, M., Veit, B. and Meyer, C. (2016) ‘TOR Signaling and Nutrient Sensing.’ Annual Review of Plant Biology, 67(1) pp. 261–285.

Dong, P., Xiong, F., Que, Y., Wang, K., Yu, L., Li, Z. and Ren, M. (2015) ‘Expression profiling and functional analysis reveals that TOR is a key player in regulating photosynthesis and phytohormone signaling pathways in Arabidopsis.’ Frontiers in Plant Science, 6(September) pp. 1–15.

Endrizzi, K., Moussian, B., Haecker, A., Levin, J. Z. and Laux, T. (1996) ‘The SHOOT MERISTEMLESS gene is required for maintenance of undifferentiated cells in Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE.’ Plant Journal pp. 967–979.

Fu, L., Liu, Y., Qin, G., Wu, P., Zi, H., Xu, Z., Zhao, X., Wang, Y., Li, Y., Yang, S., Peng, C., Wong, C. L., Yoo, S. D., Zuo, Z., Liu, R., Cho, Y. H. and Xiong, Y. (2021) ‘The TOR–EIN2 axis mediates nuclear signalling to modulate plant growth.’ Nature. Springer US, 591(7849) pp. 288–292.

Garcês, H. M. P., Champagne, C. E. M., Townsley, B. T., Park, S., Malhó, R., Pedroso, M. C., Harada, J. J. and Sinha, N. R. (2007) ‘Evolution of asexual reproduction in leaves of the genus Kalanchoë.’ Proceedings of the National Academy of Sciences of the United States of America, 104(39) pp. 15578–15583.

Garcês, H. M. P., Koenig, D., Townsley, B. T., Kim, M. and Sinha, N. R. (2014) ‘Truncation of LEAFY COTYLEDON1 protein is required for asexual reproduction in Kalanchoë daigremontiana.’ Plant Physiology, 165(1) pp. 196–206.

Garcês, H. M. P., Spencer, V. M. R. and Kim, M. (2016) ‘Control of Floret Symmetry by RAY3, SvDIV1B, and SvRAD in the Capitulum of Senecio vulgaris.’ Plant Physiology, 171(3) pp. 2055–2068.

Garcês, H. and Sinha, N. (2009) ‘The “Mother of Thousands” (Kalanchoë daigremontiana): A Plant Model for Asexual Reproduction and CAM Studies.’ Cold Spring Harbor Protocols, 2009 10 p. pbd.emo133.
Garcés, H. and Sinha, N. (2009) ‘Transformation of the plant Kalanchoë daigremontiana using Agrobacterium tumefaciens.’ Cold Spring Harbor Protocols, 4(10) pp. 4–7.

Johnson, M. A. (1934) ‘The Origin of the Foliar Pseudo-Bulbs in Kalanchoe daigremontiana.’ Bulletin of the Torrey Botanical Club, 61(7) pp. 355–366.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018) ‘MEGA X: Molecular evolutionary genetics analysis across computing platforms.’ Molecular Biology and Evolution, 35(6) pp. 1547–1549.

Kumari, A., Ray, K., Sadhna, S., Pandey, A. K., Sreelakshi, Y. and Sharma, R. (2017) ‘Metabolomic homeostasis shifts after callus formation and shoot regeneration in tomato.’ PLoS ONE, 12(5) pp. 1–26.

Lee, K. and Seo, P. J. (2017) ‘Arabidopsis TOR signaling is essential for sugar-regulated callus formation.’ Journal of Integrative Plant Biology, 59(10) pp. 742–746.

Li, L., Song, Y., Wang, K., Dong, P., Zhang, X., Li, F., Li, Z. and Ren, M. (2015) ‘TOR-inhibitor insensitive-1 (TRIN1) regulates cotyledons greening in Arabidopsis.’ Frontiers in Plant Science, 6(October) pp. 1–13.

Li, X., Cai, W., Liu, Y., Li, H., Fu, L., Liu, Z., Xu, L., Liu, H., Xu, T. and Xiong, Y. (2017) ‘Differential TOR activation and cell proliferation in Arabidopsis root and shoot apaxes.’ Proceedings of the National Academy of Sciences of the United States of America, 114(10) pp. 2765–2770.

Liu, C., Zhu, C. and Zeng, H. M. (2016) ‘Key KdSOC1 gene expression profiles during plantlet morphogenesis under hormone, photoperiod, and drought treatments.’ Genetics and Molecular Research, 15(1) pp. 1–14.

Liu, Q., Xu, C., Kirubakaran, S., Zhang, X., Hur, W., Liu, Y., Kwiatkowski, N. P., Wang, J., Westover, K. D., Gao, P., Ercan, D., Niepel, M., Thoreen, C. C., Kang, S. A., Patricelli, M. P., Wang, Y., Tupper, T., Altalabef, A., Kawamura, H., Held, K. D., Chou, D. M., Elledge, S. J., Janne, P. A., Wong, K. K., Sabatini, D. M. and Gray, N. S. (2013) ‘Characterization of Torin2, an ATP-competitive inhibitor of mTOR, ATM, and ATR.’ Cancer Research, 73(8) pp. 2574–2586.

Lotan, T., Ohto, M. A., Matsudaira Yee, K., West, M. A. L., Lo, R., Kwong, R. W., Yamagishi, K.,
Fischer, R. L., Goldberg, R. B. and Harada, J. J. (1998) ‘Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells.’ *Cell*, 93(7) pp. 1195–1205.

McCready, K., Spencer, V. and Kim, M. (2020) ‘The Importance of TOR Kinase in Plant Development.’ *Frontiers in Plant Science*, 11(February) p. 16.

Menand, B., Desnos, T., Nussaume, L., Bergert, F., Bouchez, D., Meyer, C. and Robaglia, C. (2002) ‘Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene.’ *Proceedings of the National Academy of Sciences of the United States of America*, 99(9) pp. 6422–6427.

Mohammed, B., Bilooei, S. F., Dóczi, R., Grove, E., Railo, S., Palme, K., Ditengou, F. A., Bögre, L. and López-Juez, E. (2018) ‘Converging light, energy and hormonal signaling control meristem activity, leaf initiation, and growth.’ *Plant Physiology*, 176(2) pp. 1365–1381.

Montané, M. H. and Menand, B. (2013) ‘ATP-competitive mTOR kinase inhibitors delay plant growth by triggering early differentiation of meristematic cells but no developmental patterning change.’ *Journal of Experimental Botany*, 64(14) pp. 4361–4374.

Moreau, M., Azzopardi, M., Clément, G., Dobrenel, T., Marchive, C., Renne, C., Martin-Magniette, M. L., Taconnat, L., Renou, J. P., Robaglia, C. and Meyer, C. (2012) ‘Mutations in the Arabidopsis homolog of LST8/GβL, a partner of the target of Rapamycin kinase, impair plant growth, flowering, and metabolic adaptation to long days.’ *Plant Cell*, 24(2) pp. 463–481.

Nikovics, K., Blein, T., Peaucelle, A., Ishida, T., Morin, H., Aida, M. and Laufs, P. (2006) ‘The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis.’ *Plant Cell*, 18(11) pp. 2929–2945.

Pfeiffer, A., Janocha, D., Dong, Y., Medzihradszky, A., Schöne, S., Daum, G., Suzaki, T., Forner, J., Langenecker, T., Rempel, E., Schmid, M., Wirtz, M., Hell, R. and Lohmann, J. U. (2016) ‘Integration of light and metabolic signals for stem cell activation at the shoot apical meristem.’ *eLife*, 5(JULY) pp. 1–12.

Ren, M., Qiu, S., Venglat, P., Xiang, D., Feng, L., Selvaraj, G. and Datla, R. (2011) ‘Target of rapamycin regulates development and ribosomal RNA expression through kinase domain in...
788 Arabidopsis.' Plant Physiology, 155(3) pp. 1367–1382.

789 Robaglia, C., Menand, B., Lei, Y., Sormani, R., Nicolaï, M., Gery, C., Teoulé, E., Deprost, D. and Meyer, C. (2004) ‘Plant growth: The translational connection.’ Biochemical Society Transactions, 32(4) pp. 581–584.

792 Sapre, S., Tiwari, S. and Thakur, V. V. (2018) ‘Phylogenetic analysis of target of rapamycin (TOR) kinase gene of some selected plants species.’ Bioscience Biotechnology Research Communications, 11(3) pp. 476–480.

795 Schepetilnikov, M., Dimitrova, M., Mancera-Martínez, E., Geldreich, A., Keller, M. and Ryabova, L. A. (2013) ‘TOR and S6K1 promote translation reinitiation of uORF-containing mRNAs via phosphorylation of eIF3h.’ EMBO Journal, 32(8) pp. 1087–1102.

798 Stecher, G., Tamura, K. and Kumar, S. (2020) ‘Molecular evolutionary genetics analysis (MEGA) for macOS.’ Molecular Biology and Evolution, 37(4) pp. 1237–1239.

801 Stone, S. L., Kwong, L. W., Yee, K. M., Pelletier, J., Lepiniec, L., Fischer, R. L., Goldberg, R. B. and Harada, J. J. (2001) ‘LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development.’ Proceedings of the National Academy of Sciences of the United States of America, 98(20) pp. 11806–11811.

804 De Vleesschauwer, D., Filipe, O., Hoffman, G., Seifi, H. S., Haeck, A., Canlas, P., Van Bockhaven, J., De Waele, E., Demeestere, K., Ronald, P. and Hofte, M. (2018) ‘Target of rapamycin signaling orchestrates growth–defense trade-offs in plants.’ New Phytologist, 217(1) pp. 305–319.

808 Wang, P., Zhao, Y., Li, Z., Tao, W. A., Wang, P., Zhao, Y., Li, Z., Hsu, C., Liu, X., Fu, L. and Hou, Y. (2018) ‘Reciprocal Regulation of the TOR Kinase and ABA Receptor Balances Plant Growth and Stress Response.’ Molecular Cell. Elsevier Inc., 69(1) pp. 100-112.e6.

812 Weigel, D. and Glazebrook, J. (2002) Arabidopsis: a laboratory manual. Cold Spring Harbor Laboratory Press.

814 Xiong, F., Dong, P., Liu, M., Xie, G., Wang, K., Zhuo, F., Feng, L., Yang, L., Li, Z. and Ren, M. (2016) ‘Tomato FK506 binding protein 12KD (FKBP12) mediates the interaction between...
rapamycin and target of rapamycin (TOR).’ *Frontiers in Plant Science*, 7(NOVEMBER2016).

Xiong, Y., McCormack, M., Li, L., Hall, Q., Xiang, C. and Sheen, J. (2013) ‘Glucose-TOR signalling reprograms the transcriptome and activates meristems.’ *Nature*, 496(7444) pp. 181–186.

Zhu, C., Wang, L., Chen, J., Liu, C., Zeng, H. and Wang, H. (2017) ‘Over-expression of KdSOC1 gene affected plantlet morphogenesis in Kalanchoe daigremontiana.’ *Scientific Reports*. Springer US, 7(1) pp. 1–12.

Zoulias, N., Duttke, S. H. C., Garcês, H., Spencer, V. and Kim, M. (2019) ‘The Role of Auxin in the Pattern Formation of the Asteraceae Flower Head (Capitulum).’ *Plant Physiology*, 179(2) pp. 391–401.
Figure 1. Plantlet formation in Kalanchoë. A-E Kalanchoë species evolved different strategies in terms of plantlet formation. Kalanchoë thyrsiflora does not make plantlets (A), whereas Kalanchoë pinnata makes plantlets when leaves are detached (B, C). Kalanchoë daigremontiana constitutively makes plantlets along the margins of the leaves in favourable conditions (D and E). F-J, Stages of wild-type K. daigremontiana plantlet formation. F, Stage 0: young leaf indentation with no evidence of pedestal or plantlet. G, Stage 1: no pedestal has formed and the margin is visibly raised at the node. H, Stage 2: a pedestal has formed. I, Stage 3: a thin, pin-shaped plantlet emerges from pedestal which is visible with the naked eye. J, Stage 4: plantlet cotyledons begin to elongate and become rounder. Scale bars: 5 cm (A-E); 200 µm (F-J). co; cotyledon, no; node, pe; pedestal, pl; plantlets, pp; plantlet primordium.
Figure 2. Expression of KdTOR through Kalanchoë daigremontiana plantlet formation. A-G, pKdTOR::GUS lines showed GUS expression through stages 0-3 of plantlet formation. Signal was detected at the hydathode (A, arrow), the indentation of the leaf margin (B, arrow), and at the node within the indentation (C-D, arrows). At the pedestal, GUS was expressed in the initiating plantlet primordium (E) and in the early stages of cotyledon development (F), but not in the expanding cotyledons (G-I). Whilst signal was detected in the root primordia (I) and the root tips of mature plantlets (J), GUS did not accumulate in the SAM of plantlets (K-L) or mature plants (M-N). O, qRT-PCR of KdTOR expression in Stages 1-3, relative to Stage 0. One-Way ANOVA with Dunnet’s Multiple comparison, n=3. Scale bars: 1 mm (A, I, J, K, M); 0.5 mm (L, N); 200 µm (B-H). co; cotyledon, pe; pedestal, pp; plantlet primordium, rp; root primordium, ro; root, lp; leaf primordium, SAM; shoot apical meristem.
Figure 3. Torin2, AZD-8055 and mock treatment of Kalanchoë daigremontiana. A and B, Plantlets planted on media containing 100 µM Torin2 had significantly impaired growth. In Torin2 treated plants, total leaf area was significantly lower than mock from day 7 (A) and leaf number was significantly lower from day 21 (B) until the final day of measurement (day 29). C-F, 100 µM Torin2 brushed directly onto the leaf margins reduced plantlet formation. Mock and Torin2 treated leaves longer than 3 cm had a similar percentage of plantlet formation throughout the time course (C), however leaves shorter than 3 cm had delayed plantlet formation between days 3 and 24 after Torin2 treatment (D). Both leaf sizes had a significant overall reduction in the number of plantlets per leaf after Torin2 treatment (E-F), which was markedly lower from day 12 for leaves shorter than 3 cm (F). G-J, 2 µM, 20 µM and 40 µM AZD-8055 brushed directly onto the leaf margins reduced plantlet formation independent of leaf size. Percentage of plantlet formation in AZD-8055 treated leaves longer than 3 cm was markedly lower between days 7 and 21, 7 and 28, and 7 and 35 at AZD8055 concentrations of 2 µM, 40 µM and 20 µM, respectively (G). Leaves shorter than 3 cm had slightly reduced percentage of plantlet formation between days 14 and 21 when treated with 2 µM and 20 µM AZD-8055 (H). Mean number of plantlets was significantly lower in leaves longer than 3 cm (I) and shorter than 3 cm (J) treated with 2 µM, 20 µM and 40 µM AZD-8055. There was no significant difference in mean plantlet number between AZD-8055 concentrations. Error bars show SEM. Two Way ANOVA (Repeated Measures) with Sidak’s Multiple Comparisons tests (95 % Confidence limits) (A, B, E, F) and Least Squares Mean with Tukey's P value adjustment (95 % Confidence limits) (E, F, I, J).
Figure 4. Genotypic analysis and phyllotaxy phenotypes of 35S::KdTORa silencing lines. A-C, PCR confirms 35S::KdTORa lines are transgenic and KdTOR is downregulated. Amplification of transgene (A) and NPTII (B) confirmed the transgene was present in independent lines, and quantitative RT-PCR of KdTOR expression in independent 35S::KdTORa lines showed that expression is reduced (C). Negative control (-): wild type; positive control (+): KdTORa::pBI128 plasmid. Kd18S is used as a control for C. D-G, Whole plant phenotypes in 35S::KdTORa lines. D-G, Wild-type K. daigremontiana leaves emerge in pairs in an opposite and decussate phyllotactic order (D). After the two leaf primordia emerge (L1 and L2, E), a hollow tube-like structure is formed (F), before growth of the two equally sized leaves (G). H-K, 35S::KdTORa leaves can emerge three at a time from the same node (H). Three similarly sized leaves can be detected from emergence to expansion (I-K). L-O, 35S::KdTORa leaves can emerge in an alternate phyllotactic order (L), forming one leaf at a time (M). Consequently, the older leaves (L3, L2) are larger than the younger leaves (L1) (N, O). Scale bars: 1 cm (D, H, L, G, K, O); 400 μm (E, I, M); 1 mm (F, J, N). L1; Leaf 1, L2; Leaf 2, L3; Leaf 3.
Figure 5. Phenotype analysis of plantlets in *Kalanchoë daigremontiana 35S::KdTORa* silencing lines. A-D, *KdTOR* silencing significantly reduced plantlet formation. In wild-type *K. daigremontiana*, plantlets are produced along the leaf margins (A). As illustrated in lines A (B) and H (C), all transgenic lines had significantly fewer plantlets per leaf (*P* Value <0.0001) than wild type (D). E-H, *KdTOR* silencing significantly reduced indentation of the leaf margin. Wild-type *K. daigremontiana* have regular leaf margin indentations where plantlets form (E). Margins of *KdTOR* silencing lines appeared smooth and irregular (F, G), and had significantly fewer indentations (*P* value <0.0001) in the leaf margins than wild type (H). I-L, The indentations of *KdTOR* leaves were rarely occupied by a plantlet. In wild-type plants, nearly all indentations along the leaf margin are occupied by a plantlet (I), whereas transgenic plants have a lower percentage of indentations occupied (L) with a plantlet (J) than without (K). One-Way ANOVA with Dunnett’s Multiple comparison. Scale bars: 1 cm.
Figure 6. Defective 35S::KdTORa plantlet development at the indentation. A-L, KdTOR silencing lines display defects in plantlet development at all stages of plantlet formation. The tissue rises at the node in wild type at stage 1 (A), however, in some KdTOR silencing lines, raised tissues at the node were aborted or became necrotic (B). Compared to wild type (C, E), the pedestal was misshapen and not visible (stage 2, D), or plantlet cotyledons emerging from the pedestal were misshapen and exposed on the shortened pedestal (Stage 3, F) in transgenic lines. Whilst the two wild type cotyledons at stage 4 were beginning to round (G), the larger cotyledon of the transgenic plantlet often developed a bilobed shape (H). If the transgenic cotyledons did not become chlorotic and shrivelled (J), they retained their bilobed shape at maturity (L). M-T, Scanning electron microscope images of wild type (M-P) and 35S::KdTORa indentations (Q-T), showing Stage 1 (M, Q), 3 (N, O, R, S) and 4 (P, T) plantlets. In transgenic plants, plantlet formation aborted prior to pedestal formation (Q) or terminated at the pedestal, leaving necrotic tissue (R) or non-viable plantlet structures (S, T). Scale bars: 200 µm (A-J; M-T); 1 cm (K-L).
Figure 7. qRT-PCR analysis of 35S::KdTORa silencing lines. A-D, qRT-PCR of KdSTM, KdLEC1, KdJAG and KdCUC2 expression in 35S::KdTORa silencing lines, relative to wild type (WT). Expression levels of KdSTM (A) and KdLEC1 (B), two known regulators for plantlet formation, and KdJAG (C) and KdCUC2 (D) controlling leaf crenulation were decreased in the 35S::KdTORa silencing lines. E-H, qRT-PCR of known TOR downstream genes, KdRAP2.6L, KdS5, KdPCNA1 and KdEBP which showed similar expression patterns to those of TOR inhibited Arabidopsis plants. Kd18S was used as control. One-Way ANOVA with Dunnet’s Multiple comparison, n=3.
Figure 8. Auxin treatment of \textit{pKdTOR::GUS} lines to test upstream activation of \textit{KdTOR}. \textit{pKdTOR::GUS} leaves were incubated in mock solution (A-E), 25 µM IAA (F-J), or 25 µM NPA (K-O) for 24 hours. There were no differences in expression in the hydathodes (stage 0; A, F, K), in the plantlet primordia (stage 2; B, G, L), or in the developing cotyledons (stage 3; C, H, M). However, roots of plantlets treated with IAA had stronger \textit{GUS} expression in the epidermis and cortex of the elongation zone (I-J) when compared to mock (D, E) or NPA treated (N, O) roots. Scale bars: 500 µm (A, F, K); 100 µm (B, G, L); 200 µm (C-E, H-J, M-O).
Barrada, A., Djendli, M., Desnos, T., Mercier, R., Robaglia, C., Montané, M. H. and Menand, B. (2019) 'A TOR-YAK1 signaling axis controls cell cycle, meristem activity and plant growth in Arabidopsis.' Development (Cambridge, England), 146(3).

Batygina, T. B., Bragina, E. A. and Titova, G. E. (1996) 'Morphogenesis of Propagules in Viviparous Species Bryophyllum Daigremontianum and B. Calycinum.' Acta Societatis Botanicorum Poloniae, 65(1–2) pp. 127–133.

Bilsborough, G. D., Runions, A., Barkoulas, M., Jenkins, H. W., Hasson, A., Galinha, C., Laufs, P., Hay, A., Prusinkiewicz, P. and Tsiantis, M. (2011) 'Model for the regulation of Arabidopsis thaliana leaf margin development.' Proceedings of the National Academy of Sciences of the United States of America, 108(8) pp. 3424–3429.

Braybrook, S. A. and Harada, J. J. (2008) 'LECs go crazy in embryo development.' Trends in Plant Science, 13(12) pp. 624–630.

Chaiwanon, J., Wang, W., Zhu, J. Y., Oh, E. and Wang, Z. Y. (2016) 'Information Integration and Communication in Plant Growth Regulation.' Cell. Elsevier Inc., 164(6) pp. 1257–1268.

Chen, G. H., Liu, M. J., Xiong, Y., Sheen, J. and Wu, S. H. (2018) 'TOR and RPS6 transmit light signals to enhance protein translation in deetiolating Arabidopsis seedlings.' Proceedings of the National Academy of Sciences of the United States of America, 115(50) pp. 12823–12828.

Chresta, C. M., Davies, B. R., Hickson, I., Harding, T., Cosulich, S., Critchlow, S. E., Vincent, J. P., Ellston, R., Jones, D., Sini, P., James, D., Howard, Z., Dudley, P., Hughes, G., Smith, L., MaQUIRE, S., Hummersone, M., Malagu, K., Menear, K., Jenkins, R., Jacobsen, M., Smith, G. C. M., Guichard, S. and Pass, M. (2010) 'AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity.' Cancer Research, 70(1) pp. 288–298.

Deprost, D., Deprost, D., Yao, L., Sormani, R., Moreau, M., Leterreux, G., Bedu, M., Robaglia, C. and Meyer, C. (2005) 'An Arabidopsis homolog of RAPTOR/KOG1 is essential for early embryo development.' Biochemical and Biophysical Research Communications, 326(4) pp. 844–850.

Deprost, D., Yao, L., Sormani, R., Moreau, M., Leterreux, G., Bedu, M., Robaglia, C. and Meyer, C. (2007) 'The Arabidopsis TOR kinase links plant growth, yield, stress resistance and mRNA translation.' EMBO Reports, 8(9) pp. 864–870.

Dinneny, J. R., Yadegari, R., Fischer, R. L., Yanofsky, M. F. and Weigel, D. (2004) 'The role of JAGGED in shaping lateral organs.' Development, 131(5) pp. 1101–1110.

Dobrenel, T., Caldana, C., Hanson, J., Robaglia, C., Vincentz, M., Veit, B. and Meyer, C. (2016) 'TOR Signaling and Nutrient Sensing.' Annual Review of Plant Biology, 67(1) pp. 261–285.

Dong, P., Xiong, F., Que, Y., Wang, K., Yu, L., Li, Z. and Ren, M. (2015) 'Expression profiling and functional analysis reveals that TOR is a key player in regulating photosynthesis and phytohormone signaling pathways in Arabidopsis.' Frontiers in Plant Science, 6(september) pp. 1–15.

Endrizzi, K., Moussian, B., Haecker, A., Levin, J. Z. and Laux, T. (1996) 'The SHOOT MERistemLESS gene is required for maintenance of undifferentiated cells in Arabidopsis shoot and floral meristems and acts at a different regulatory level than the meristem genes WUSCHEL and ZWILLE.' Plant Journal pp. 967–979.

Fu, L., Liu, Y., Qin, G., Wu, P., Zi, H., Xu, Z., Zhao, X., Wang, Y., Li, Y., Yang, S., Peng, C., Wong, C. C. L., Yoo, S. D., Zuo, Z., Liu, R., Cho, Y. H. and Xiong, Y. (2021) 'The TOR–EIN2 axis mediates nuclear signalling to modulate plant growth.' Nature. Springer US, 591(7849) pp. 288–292.

Garcés, H. M. P., Champagne, C. E. M., Townsley, B. T., Park, S., Malhó, R., Pedrosa, M. C., Harada, J. J. and Sinha, N. R. (2007) 'Evolution of asexual reproduction in leaves of the genus Kalanchoë.' Proceedings of the National Academy of Sciences of the United States of America, 104(39) pp. 15578–15583.

Garcés, H. M. P., Koenig, D., Townsley, B. T., Kim, M. and Sinha, N. R. (2014) 'Truncation of LEAFY COTYLEDON1 protein is
required for asexual reproduction in Kalanchoë daigremontiana.' Plant Physiology, 165(1) pp. 196–206.

Garcês, H. M. P., Spencer, V. M. R. and Kim, M. (2016) 'Control of Floret Symmetry by RAY3, SvDIV1B, and SvRAD in the Capitulum of Senecio vulgaris.' Plant Physiology, 171(3) pp. 2055–2068.

Garcês, H. and Sinha, N. (2009) 'The "Mother of Thousands" (Kalanchoë daigremontiana): A Plant Model for Asexual Reproduction and CAM Studies.' Cold Spring Harbor Protocols, 2009 10 p. pbd.emo133.

Garcês, H. and Sinha, N. (2009) 'Transformation of the plant Kalanchoë daigremontiana using Agrobacterium tumefaciens.' Cold Spring Harbor Protocols, 4(10) pp. 4–7.

Johnson, M. A (1934) 'The Origin of the Foliar Pseudo-Bulbils in Kalanchoe daigremontiana.' Bulletin of the Torrey Botanical Club, 61(7) pp. 355–366.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018) 'MEGA X: Molecular evolutionary genetics analysis across computing platforms.' Molecular Biology and Evolution, 35(6) pp. 1547–1549.

Kumari, A., Ray, K., Sadhna, S., Pandey, A. K., Sreelakshmi, Y. and Sharma, R. (2017) 'Metabolomic homeostasis shifts after callus formation and shoot regeneration in tomato.' PLoS ONE, 12(5) pp. 1–26.

Lee, K. and Seo, P. J. (2017) 'Arabidopsis TOR signaling is essential for sugar-regulated callus formation.' Journal of Integrative Plant Biology, 59(10) pp. 742–746.

Li, L., Song, Y., Wang, K., Dong, P., Zhang, X., Li, F., Li, Z. and Ren, M. (2015) 'TOR-inhibitor insensitive-1 (TRIN1) regulates cotyledons greening in Arabidopsis.' Frontiers in Plant Science, 6(October) pp. 1–13.

Li, X., Cai, W., Liu, Y., Li, H., Fu, L., Liu, Z., Xu, L., Liu, H., Xu, T. and Xiong, Y. (2017) 'Differential TOR activation and cell proliferation in Arabidopsis root and shoot apexes.' Proceedings of the National Academy of Sciences of the United States of America, 114(10) pp. 2765–2770.

Liu, C., Zhu, C. and Zeng, H. M. (2016) 'Key KdSOC1 gene expression profiles during plantlet morphogenesis under hormone, photoperiod, and drought treatments.' Genetics and Molecular Research, 15(1) pp. 1–14.

Liu, Q., Xu, C., Kirubakaran, S., Zhang, X., Hur, W., Liu, Y., Kwiatkowski, N. P., Wang, J., Westover, K. D., Gao, P., Erkan, D., Niepel, M., Thoreen, C. C., Kang, S. A., Patricelli, M. P., Wang, Y., Tupper, T., Altabet, A., Kawamura, H., Held, K. D., Chou, D. M., Elledge, S. J., Janne, P. A., Wong, K. K., Sabatini, D. M. and Gray, N. S. (2013) 'Characterization of Torin2, an ATP-competitive inhibitor of mTOR, ATM, and ATR.' Cancer Research, 73(8) pp. 2574–2586.

Lotan, T., Ohito, M. A., Matsudaire Yee, K., West, M. A. L., Lo, R., Kwong, R. W., Yamagishi, K., Fischer, R. L., Goldberg, R. B. and Harada, J. J. (1998) 'Arabidopsis LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells.' Cell, 93(7) pp. 1195–1205.

McCready, K., Spencer, V. and Kim, M. (2020) 'The Importance of TOR Kinase in Plant Development.' Frontiers in Plant Science, 11(February) p. 16.

Menand, B., Desnos, T., Nussaume, L., Bergert, F., Bouchez, D., Meyer, C. and Robaglia, C. (2002) 'Expression and disruption of the Arabidopsis TOR (target of rapamycin) gene.' Proceedings of the National Academy of Sciences of the United States of America, 99(9) pp. 6422–6427.

Mohammed, B., Bilooei, S. F., Dóczi, R., Grove, E., Railo, S., Palme, K., Ditengou, F. A., Bögre, L. and López-Juez, E. (2018) 'Converging light, energy and hormonal signaling control meristem activity, leaf initiation, and growth.' Plant Physiology, 176(2) pp. 1365–1381.

Montané, M. H. and Menand, B. (2013) 'ATP-competitive mTOR kinase inhibitors delay plant growth by triggering early
differentiation of meristematic cells but no developmental patterning change.' Journal of Experimental Botany, 64(14) pp. 4361–4374.

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Moreau, M., Azzopardi, M., Clément, G., Dobrenel, T., Marchive, C., Renne, C., Martin-Magniette, M. L., Taconnat, L., Renou, J. P., Robaglia, C. and Meyer, C. (2012) 'Mutations in the Arabidopsis homolog of LST8/GβL, a partner of the target of Rapamycin kinase, impair plant growth, flowering, and metabolic adaptation to long days.' Plant Cell, 24(2) pp. 463–481.

Google Scholar: Author Only Title Only Author and Title

Nikovics, K., Blein, T., Peaucelle, A., Ishida, T., Morin, H., Aida, M. and Laufs, P. (2006) 'The balance between the MIR164A and CUC2 genes controls leaf margin serration in Arabidopsis.' Plant Cell, 18(11) pp. 2929–2945.

Google Scholar: Author Only Title Only Author and Title

Pfeiffer, A., Janocha, D., Dong, Y., Medzihradsky, A., Schöne, S., Daun, G., Suzaki, T., Forner, J., Langenecker, T., Rempel, E., Schmid, M., Wirtz, M., Hell, R. and Lohmann, J. U. (2016) 'Integration of light and metabolic signals for stem cell activation at the shoot apical meristem.' eLife, 5(JULY) pp. 1–12.

Google Scholar: Author Only Title Only Author and Title

Ren, M., Qiu, S., Venglat, P., Xiang, D., Feng, L., Selvaraj, G. and Datla, R. (2011) 'Target of rapamycin regulates development and ribosomal RNA expression through kinase domain in Arabidopsis.' Plant Physiology, 155(3) pp. 1367–1382.

Google Scholar: Author Only Title Only Author and Title

Sapre, S., Tiwari, S. and Thakur, V. V. (2018) 'Phylogenetic analysis of target of rapamycin (TOR) kinase gene of some selected plants species.' Bioscience Biotechnology Research Communications, 11(3) pp. 476–480.

Google Scholar: Author Only Title Only Author and Title

Schepetilnikov, M., Dimitrova, M., Mancera-Martínez, E., Geldreich, A., Keller, M. and Ryabova, L. A. (2013) 'TOR and S6K1 promote translation reinitiation of uORF-containing mRNAs via phosphorylation of eIF3h.' EMBO Journal, 32(8) pp. 1087–1102.

Google Scholar: Author Only Title Only Author and Title

Stecher, G., Tamura, K. and Kumar, S. (2020) 'Molecular evolutionary genetics analysis (MEGA) for macOS.' Molecular Biology and Evolution, 37(4) pp. 1237–1239.

Google Scholar: Author Only Title Only Author and Title

Stone, S. L., Kwong, L. W., Yee, K. M., Pelletier, J., Lepiniec, L., Fischer, R. L., Goldberg, R. B. and Harada, J. J. (2001) 'LEAFY COTYLEDON2 encodes a B3 domain transcription factor that induces embryo development.' Proceedings of the National Academy of Sciences of the United States of America, 98(20) pp. 11806–11811.

Google Scholar: Author Only Title Only Author and Title

Weigel, D. and Glazebrook, J. (2002) Arabidopsis: a laboratory manual. Cold Spring Harbor Laboratory Press.

Google Scholar: Author Only Title Only Author and Title

Xiong, F., Dong, P., Liu, M., Xie, G., Wang, K., Zhuo, F., Feng, L., Yang, L., Li, Z. and Ren, M. (2016) 'Tomato FK506 binding protein 12KD (FKBP12) mediates the interaction between rapamycin and target of rapamycin (TOR).' Frontiers in Plant Science, 7(NOVEMBER2016).

Google Scholar: Author Only Title Only Author and Title

Xiong, Y., McCormack, M., Li, L., Hall, Q., Xiang, C. and Sheen, J. (2013) 'Glucose-TOR signalling reprograms the transcriptome and activates meristems.' Nature, 496(7444) pp. 181–186.

Google Scholar: Author Only Title Only Author and Title

Zhu, C., Wang, L., Chen, J., Liu, C., Zeng, H. and Wang, H. (2017) 'Over-expression of KdSOC1 gene affected plantlet morphogenesis in Kalanchee daigremontiana.' Scientific Reports. Springer US, 7(1) pp. 1–12.

Google Scholar: Author Only Title Only Author and Title

Zoulias, N., Duttke, S. H. C., García, H., Spencer, V. and Kim, M. (2019) 'The Role of Auxin in the Pattern Formation of the Asteraceae Flower Head (Capitulum).’ Plant Physiology, 179(2) pp. 391–401.
