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RESEARCH PAPER

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# Ectopic expression of the transcription factor CUC2 restricts growth by cell cycle inhibition in *Arabidopsis* leaves

Xiaoyu Li [a,b*], Yucai Zheng [a,b*], Qian Xing [a,b*], Rhomi Ardiansyah [a,b], Hui Zhou [c], Shahid Ali [b], Tingting Jing [a,b], Jingjing Tian [a,b], Xing Shun Song [a,c], Yuhua Li [a], and Ralf Müller-Xing [a,b]

[a]Key Laboratory of Saline-Alkali Vegetation Ecology Restoration (Northeast Forestry University), Ministry of Education, Harbin, PR China; [b]Plant Epigenetics and Development, Institute of Genetics, College of Life Science, Northeast Forestry University, Harbin, PR China; [c]Plant Genetics, Institute of Genetics, College of Life Science, Northeast Forestry University, Harbin, PR China

**ABSTRACT**

Plant leaf margins produce small outgrowths or teeth causing serration in a regular arrangement, which is specified by auxin maxima. In *Arabidopsis*, the spatiotemporal pattern of auxin dependents on both, the transcription factor CUC2 and the signal peptide EPFL2, a ligand of the growth-promoting receptor kinase ERECTA (ER). Ectopic expression of *CUC2* can have contrary effects on leaf growth. Ubiquitous expressed *CUC2* suppresses growth in the whole leaf, whereas *cuc2-1D* mutants have enlarged leaves, through *ER*-dependent cell proliferation in the teeth. Here we investigated the growth dynamics of *cuc2-1D* leaves and the growth restricting the function of *CUC2* using the ubiquitous inducible *CUC2-GR* transgene. In time courses, we dissected the serration promoting the function of *CUC2* in the leaf margin and ectopic growth inhibition by *CUC2* in the leaf plate. We found that *CUC2* limits growth rather by cell cycle inhibition than by cell size control. Furthermore, endogenous *CUC2* was rapidly induced by *CUC2-GR* indicating a possible auto-inducible feedback. In contrast, *EPFL2* was quickly decreased by transient *CUC2* induction but increased in *cuc2-3* mutant leaves suggesting that *CUC2* can also counteract the *EPFL2-ER* pathway. Therefore, tooth growth promotion and growth inhibition by *CUC2* involve partially the same mechanism but in contrary ways.

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*Arabidopsis thaliana*; leaf development; leaf growth; leaf margin; cell division; signal transduction; transcriptional regulation

## 1. Introduction

Plant leaves evolved to capture light and carbon dioxide for photosynthesis, while at the same time managing water loss and tissue temperatures within an optimum range. The leaves of *Arabidopsis thaliana* (*Arabidopsis*) are typical for dicotyledonous plants, their leaf blades are broad and thin and are held in a horizontal plane by the leaf petiole.[1] Based on the results of more than three decades of research in *Arabidopsis*, several pathways are known controlling leaf growth and shape but we are far from understanding all interconnections of the components or the complexity of the gene networks shaping the leaf. Leaves are established at the flank of the shoot apical meristem (SAM) rising from a few founder cells that are distinguished by high auxin concentrations and downregulation of the KNOTTED-like homeodomain class I (KNOXI) transcription factors KNAT1, KNAT2 (for knotted-like from *Arabidopsis thaliana*) and SHOOT MERISTEMLESS (STM); whereas *KNAT1* misexpression induces lobed leaves.[2-5] In the simple leaves of *Arabidopsis*, *KNOXI* genes are widely transcriptionally and epigenetically repressed involving transcription factors such as ASYMMETRIC LEAVES 1 (AS1) and AS2[6-9] and SAWTOOTH 1 (SAW1) and SAW2.[10] In young leaf primordia, three axes are established very quickly, and subsequent growth, expansion and differentiation follow along the proximodistal, the dorsoventral and the mediolateral axis.[2] In contrary to shoots that grow from the SAM, cells that build the leaf blade come from the meristematic zone of the leaf primordia, which is located at the junction between the leaf blade and the leaf petiole in *Arabidopsis*.[11] The meristematic zone can be distinguished in two types of dividing tissues producing the leaf plate and the leaf margin, respectively.[12]

Current research of leaf shape concentrates on the role of pattern formation in the leaf margin that controls the number and the size of leaf teeth (serration) or lobes. The process of leaf serration formation is related to the leaf primordia formation in the SAM, both are dependent on auxin maxima established by auxin efflux carrier PIN-FORMED 1 (PIN1).[13] The transcription factor CUP-SHAPED COTYLEDON 2 (CUC2), expressed in the leaf sinuses, promotes the generation of PIN1-dependent auxin activity maxima while auxin represses *CUC2* expression.[14] In leaves, *CUC2* is specifically targeted by *MIR164A*, which triggers the cleavage of the *CUC2* mRNA.[15] Expression of *MIR164A*-resistant *CUC2* phenocopies the strong-serrated *mir164a* mutant leaves suggesting that higher *CUC2* expression is linked to enhance leaf serration.[16] Consistently, *cuc2-1D* mutant which carries

**CONTACT** Ralf Müller-Xing Ralf.Mueller@hhu.de; Qian Xing q.xing@nefu.edu.cn Key Laboratory of Saline-Alkali Vegetation Ecology Restoration (Northeast Forestry University), Ministry of Education, Harbin, China

*These authors contributed equally to this work.
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a point mutation in the *MIR164A*-targeting site shows both, high *CUC2* expression and stronger outgrowth leaf serrations, which is independent of *KNAT1*.[17,18] On the other hand, the constitutive misexpression of *CUC2* and its homologue *CUC1* causes strong inhibition of overall leaf growth.[15,19-21] In *cuc2-3* loss of function mutants, the auxin maxima is abolished and accompanied by less cell proliferation around the remaining tips suggesting that *CUC2* supports the outgrowth of leaf teeth by promoting cell division.[22] Additionally, *CUC2* represses growth cell-autonomously to enhance leaf serration by inhibition of growth in the sinus.[14,16] Recent findings showed that the first morphological visible event is the repression of growth at the area of *CUC2* expression, whereas the *CUC2*-dependent outgrowth of the tooth occurs later.[23]

The *EPIDERMAL PATTERNING FACTOR* (*LIKE*) (*EPF* and *EPFL*) gene family encodes plant-specific secretory peptides, which play important roles in leaf development including control of stomata density and patterning in the epidermis.[24,25] Several of the EPF peptides, including EPFL2, are ligands of the three ERECTA-family leucine-rich repeat receptor-like kinases ERECTA (ER), ERECTA-LIKE1 (ERL1) and ERL2.[26,27] *er* mutant plants display round leaves with short petioles,[28,29] whereas the phenotype of *er erl1 erl2* triple mutants is more severe with a small rosette with small, round leaves that lack petiole elongation caused by substantially reduced cell proliferation.[30,31] The binding of EPFL2 peptide to the ER family receptors is required for leaf tooth growth that is accompanied by repression of auxin response in growing leaf margins.[26]

The opinion, how *CUC2* promotes leaf serration, changes over the years several times.[23] *CUC2* has been proposed to either locally repress growth to form the leaf sinuses[16] to promote tooth outgrowth in an auxin-dependent manner[22] or in a combination of both.[14] In the latter variant, the *CUC2*-dependent growth repression occurs initially forming the leaf sinuses, whereas, during a secondary phase, *CUC2* stabilizes non-cell autonomously PIN1 locations and so indirectly auxin maxima that are required for tooth formation.[14,23] Recently, showed that *EPFL2* signaling promotes leaf tooth growth via repression of auxin response in the growing leaf margin.[26] Our study shows a more precise analysis of *cuc2-1D* and *35S::CUC2-GR* (*CUC2-GR*)[17,19] focused on the role of *CUC2* in leaf growth and leaf margin development revealing new aspects of these *Arabidopsis* plant lines and growth control by *CUC2*. Here, we provide evidences that *CUC2* inhibits growth in the sinus by controlling rather cell division than cell size. We demonstrate that growth inhibition by *CUC2* is a consequence of reduced cell division and that the spatio-temporal pattern of ectopic *CUC2* in the whole leaf blade or the leaf sinus affects leaf size and shape. Transient ubiquitous induction revealed that *CUC2* can repress *EPFL2* and therefore counteract the cell proliferation promoting *ER* signaling. Furthermore, we discuss how our findings expand the common view of *CUC2*-dependent growth regulation in leaves by including cell cycle control in the leaf plate to the margin-centered growth model.

## 2. Results

### 2.1. CUC2misexpression can have contrary effects to the growth of Arabidopsis leaves

While studying leaf serration in mutants defective in the epigenetic machinery, we used loss-of-function *cuc2-3* mutants and dominant *cuc2-1D* mutants carrying a *MIR164*-resistant *CUC2* allele, as controls, which either loses all or has

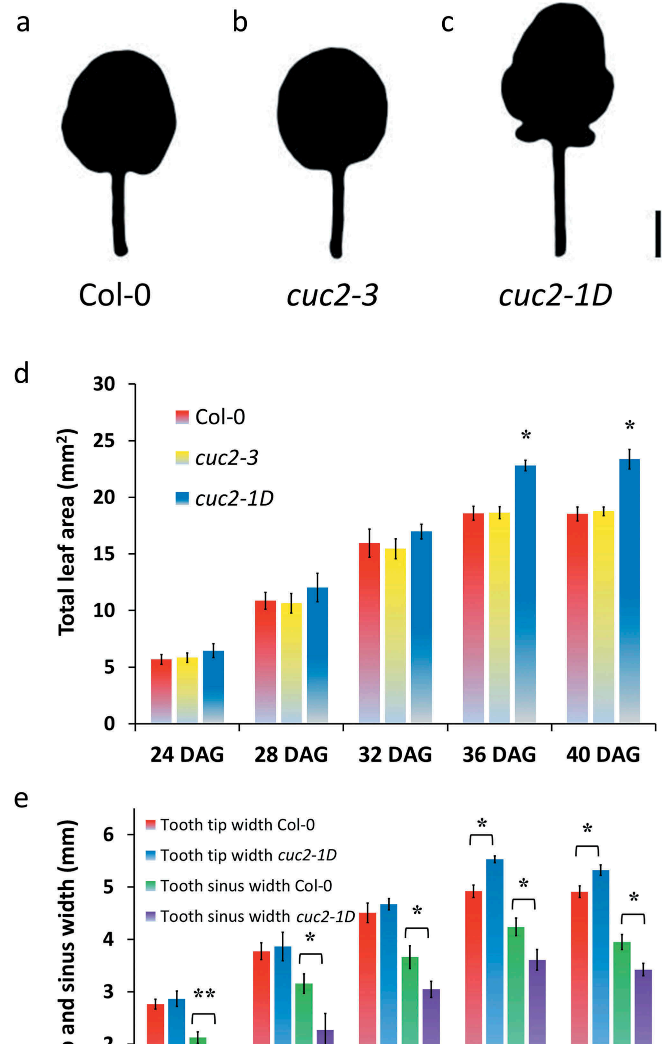


**Figure 1.** Contrary effects on leaf growth by gaining of *CUC2* function in *cuc2-1D* mutants. A-C, Silhouette of fourth rosette leaf of wild-type (Col-0) (a), *cuc2-3* (b) and *cuc2-1D* mutants (c), 40 DAG. Scale Bars = 2 mm. D, Time course of the dynamics of the total leaf area. *, Significant enlarged (Student's t-test $p < .05$) in comparison with Col-0 and *cuc2-3* mutant leaves of the same age. Note that each genotype did not show any significant difference between 36 and 40 DAG indicating the leaf growth of all three genotypes terminated around 36 DAG. E, Antagonistic dynamics of the leaf width at the 1st tooth tips and at the sinuses between the 1st and 2nd tooth of the fourth rosette leaf of Col-0 and *cuc2-1D* mutants, 40 DAG. D-E, N = 10 (24–36 DAG), N = 20 (40 DAG); ± SE. Significant changes (Student's t-test, *, $p < .05$, **, $p < .01$) between Col-0 and *cuc2-1D* mutant leaves of the same age.

enhanced leaf serration, respectively (Figure 1a–c, [32, 16, 17]). We were wondering why *cuc2-1D* mutants have a significant increased total area of rosette leaves (Figure 1a,c; [18]), whereas constitutive misexpression of *CUC2* is described as reducing leaf size.[32] In order to get a better understanding of the growth dynamics, we measured the leaf surface area of *cuc2-1D* in comparison with Col-0 and *cuc2-3* in a time course (Figure 1d). Interestingly, the leaf surface area of *cuc2-1D* was not distinguishable before all three leaf types, Col-0, *cuc2-3*, and *cuc2-1D*, stopped to grow (Figure 1d, 36 DAG). Using toothless *cuc2-3* leaves, it was shown that wild-type teeth emerge rather by the outgrowth of the leaf tooth tip than by growth repression in the sinus.[22] To prove whether the increased leaf area of *cuc2-1D* leaves is primarily caused by enhanced tooth growth, we also measured the width and the length in different areas of the leaves (Figure 1e and S1). The width between the first tooth tips and length of leaf blades and petioles was slightly increased in *cuc2-1D* mutants verifying the results from 17, which also mentioned that leaf width at the sinus of *cuc2-1D* were significantly wider than Col-0 suggesting that the increased medial-lateral expansion of *cuc2-1D* leaves was not only due to an outgrowth of leaf teeth but a general enhancement of leaf expansion. In contrast, we found a significant reduction of the leaf wide at some sinuses indicating growth inhibition inside of the leaf blade at least in the area of these sinuses (Figure 1e). This growth suppression occurred early (before 24 DAG) underneath the sinus in *cuc2-1D* leaves and was also later not covered by tooth outgrowth, which became significant 36 DAG at the very moment when total leaf growth stopped in all three leaf types (Figure 1d,e).

Our results suggest that in contrast to earlier reports misexpression of *CUC2* inhibits growth at least in some areas of *cuc2-1D* leaves. However, our findings may apply only to a subset of rosette leaves as we analyzed mainly juvenile leaves. Leaves, which rise after vegetative phase change, display stronger serrated margins.[33] That might increase medial-lateral expansion at the leaf tooth margin, which could cover the growth inhibition in the sinus but we found rather stronger growth suppression in the sinus of later rosette leaves (Figure S1D). Hence, leaf growth inhibition seems a shared feature of plants constitutively over-expressing *CUC2* and *cuc2-1D* mutants.

## 2.2. Cuc2-dependent growth suppression is not limited to early leaf stages

During early leaf development, *CUC2* is expressed along the whole margin of wild-type leaf primordia. Later, *CUC2* expression is ceased in the developing teeth and restricted to the sinus area.[14,16] When the leaf growth has stopped, *CUC2* expression is harder to observe in the sinus regions and then finally vanish [[16], Xiaoyu Li and Ralf Müller-Xing, unpublished data]. This raises the question whether leaf cells are competent to react to ectopic *CUC2* long after endogenous *CUC2* expression is terminated and earlier studies with constitutive expressed *CUC2* did not examine that matter in detail.[15,19-21] Considering our data about growth dynamics in *cuc2-1D* leaves, it seems that the spatiotemporal pattern of *CUC2* could influence whether *CUC2* promotes or suppresses growth in leaves. In order to investigate the temporal aspect, we decided to use an inducible system with an unmodified *CUC2* cDNA for our induced overexpression experiments [*CUC2-GR* friendly provided by Ben Scheres].[19] Using that genetic tool insured that the natural regulation of *CUC2* by *MIR164A* was not compromised, in contrast to earlier used microRNA resistance versions [*CUC2m*].[16,34,35,32]

We investigated carefully the consequences of starting or withdrawing continued *CUC2* overexpression at different time points (Figure 2a). First, we compared the rosette and leaf phenotype of continuously dexamethasone (DEX) treated *CUC2-GR* and Col-0 plants with untreated controls (Figures 2b–g and 3a,c). In the presence of DEX, we observed no changes in Col-0 plants but strong growth inhibition in all areal parts of *CUC2-GR* plants resembling constitutive *CUC2* expression (Figures 2b–f and 3a,c), whereas the total leaf area of induced *CUC2-GR* was reduced to 25% (Figure 3a). Interestingly, we found strong enhanced teeth in continuously induced *CUC2-GR* leaves (Figures 2f,g and 3c, S2A). We measured further growth parameters of the continuously induced *CUC2-GR* leaves and found overall inhibition of growth along the proximo-distal and the medio-lateral axes (Figure S3).

In a second approach, we started or withdraw continued *CUC2* overexpression at serial days between 10–22 DAG and found consistent gradients of increasing or decreasing the size of rosettes or leaves, respectively (Figures 2h–x and 3b,d, S2). Even starting induction or withdrawing of DEX only one or 2 days before measurement (22 DAG) had a clear impact on the total leaf area indicating that at least the same region of the leaf can react to ectopic *CUC2* in very late developmental stages.

## 2.3. CUC2-GR can initiate ectopic tooth growth only in early leaf stages

Leaves, which were exposed to high *CUC2* expression during their formation as in *cuc2-1D* or continuously induced *CUC2-GR*, show much stronger leaf serration than wild-type (Figures 1a,c, 2f–h, 3c). More in detail, we observed that an early start of continued *CUC2-GR* induction can promote growth in leaf teeth thereby the decrease of the total leaf size reached its maximum (Figures 2f–h, 3c). The teeth size of continuously DEX-treated *CUC2-GR* leaves was significant enlarged compared to the controls (Figure 3c, S2A). *CUC2-GR* leaves, with continuous DEX treatment started later than 12 DAG, were clearly smaller but did not longer display increased teeth (Figure 2i–l, S2A-B). Consistently, tooth size was also increased in *CUC2-GR* leaves initiated during DEX treatment although DEX was then early withdrawn (≥11 DAG, Figure 2s–x). Taken together, it seems that ectopic expressed *CUC2* can enhance leaf serration only in very

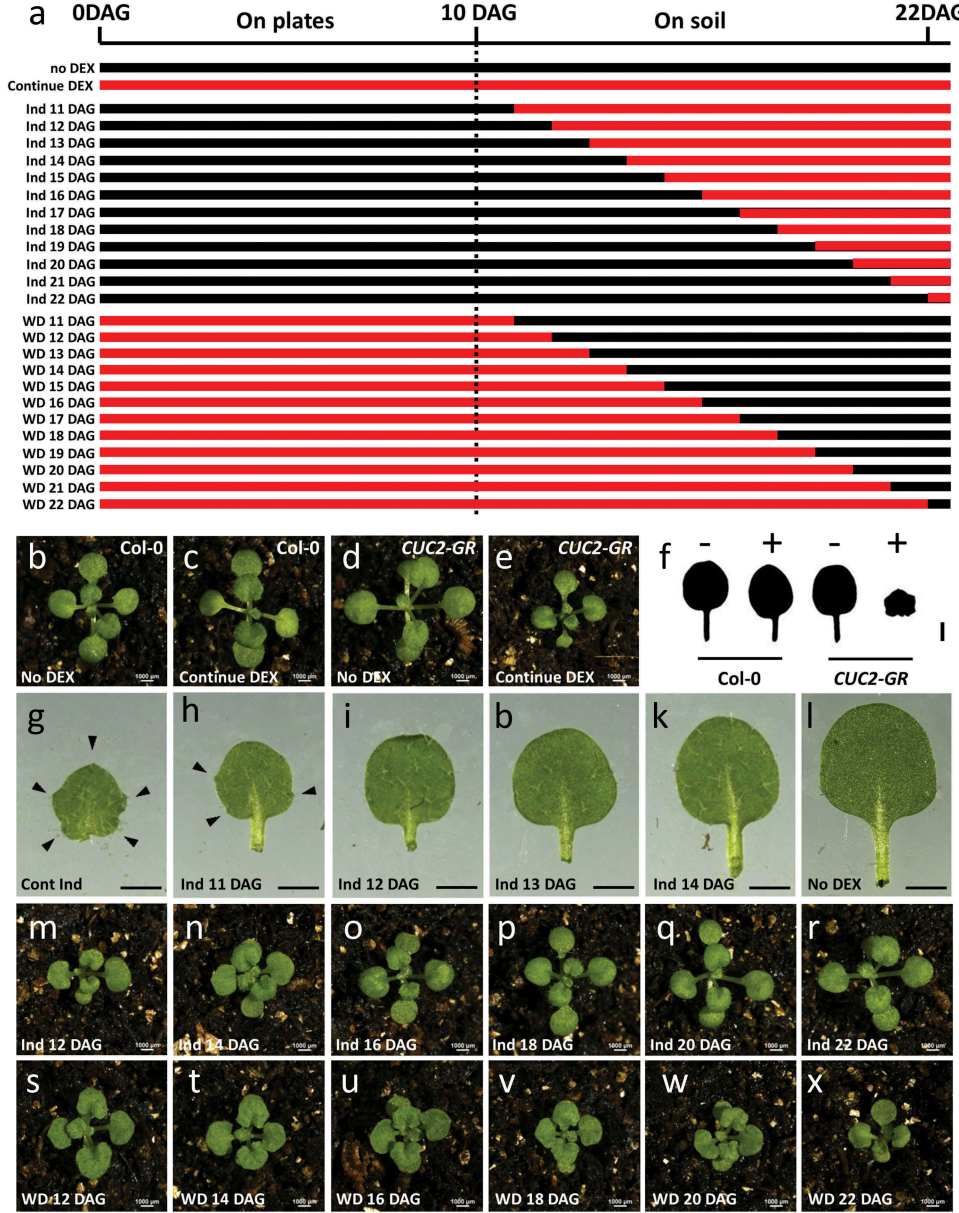


**Figure 2.** Early continued induction of *CUC2-GR* can promote growth in leaf teeth but decrease total leaf size. A, Scheme of induction time courses of DEX treatments. Red lines indicating DEX induction, black one non-treatment. B-X, All leaf phenotypes were analyzed at 22 DAG. B-E, Leaf rosettes of Col-0 after no DEX (b) and continuous DEX treatment (c), *CUC2-GR* with no DEX (d) and continuous DEX (e). F, Leaf silhouettes of the fourth rosette leaf; +, continuous DEX treatment, -, No DEX control. G, Fourth leaf continuously treated with DEX (Cont Ind). H-L, The fourth leaves were initially not treated with DEX, but continuous DEX-induced (ind) starting from 11 DAG (h), 12 DAG (i), 13 DAG (j), 14 DAG (k), No DEX control (l). M-R, Plants were initially not treated with DEX but continuous DEX-induced (ind) starting from 12 DAG (m), 14 DAG (n), 16 DAG (o), 18 DAG (p), 20 DAG (q) and 22 DAG (r) on. S-X, From the germination on, plants were treated continuously with DEX but DEX was withdrew (WD) from 12 DAG (s), 14 DAG (t), 16 DAG (u), 18 DAG (v), 20 DAG (w) and 22 DAG (x) on. Scale bars (b-x) = 1000 µm.

early leaf stages, whereas can inhibit the overall growth of the leaf blade also in later stages.

## 2.4. *CUC2* represses several key regulators of leaf development but induces its own expression

Leaf growth and patterning involve coordinated regulation among transcription factors[36] that build several partially interconnected networks. To test which down-stream transcription factors react rapidly to induced *CUC2-GR*, we performed quantitative RT-PCR analyses (qRT-PCRs; Figure 4) within 3 h after DEX induction (3 HAI) focusing on transcription factors that are involved in both growth regulation and formation of leaf serration and are predicted to work upstream or downstream of *CUC2*.

In many plant species with strong serrated or compound leaves, *KNOXI* genes are expressed in the leaf sinus that plays a fundamental role in the serration process.[37,38] Furthermore, the *KNOX* gene *STM* is misexpressed in the sinus of older leaves of plants carrying the *CUC2g-m4* transgene, which is *MIR164A*-resistant resembling *cuc2-1D* mutants.[22] Although the ectopic *STM* expression correlates rather with ectopic meristem formation than early *CUC2* misexpression.[22] To test whether the *KNOXI* genes response to temporary increased *CUC2* activity, we checked their mRNA abundance in *CUC2-GR* leaves by qRT-PCR 3 HAI with DEX. Interestingly, the mRNA levels of *KNAT1*,

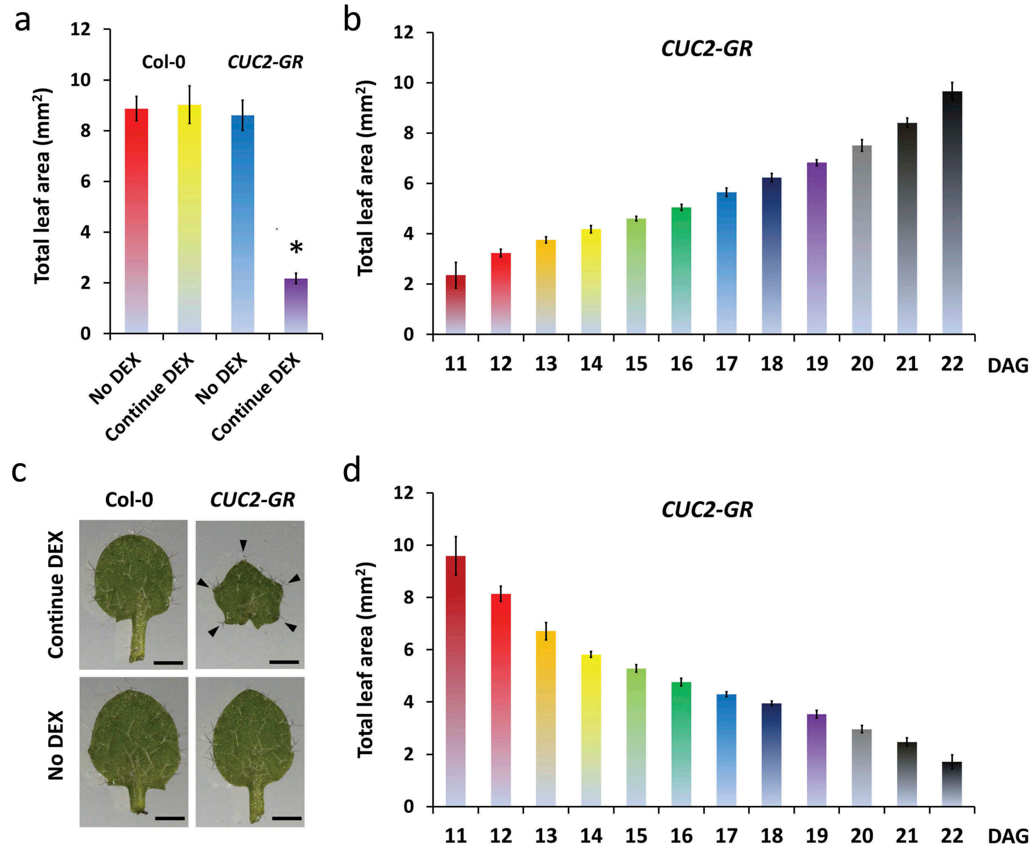


**Figure 3.** The effects of *CUC2-GR* on leaf growth correlate with the length of DEX treatment. A, C-D, Total leaf area of the fourth leaf of non-treated, temporary and continuous DEX-treated *Arabidopsis* plants; all leaves were measured at 22 DAG; N = 10, ± SE. A, Continuous DEX-treatment Col-0 and *CUC2-GR* leaves in comparison to non-treated. * Significant difference compared to the others (Student's t-test $p < .05$) B, DEX-induction time course: The continuous DEX-treatment started between 11 DAG and 22 DAG. C, Fourth leaves of continuous DEX-treated (started 0 DAG) and non-treated Col-0 and *CUC2-GR* plants, 16 DAG. D, DEX-withdrew time course: The DEX-treatment was stopped between 11 DAG and 22 DAG. Note that there was no significant difference of the leaf areas between continuous DEX (a), continued DEX induction started at 11 DAG (b) and DEX withdrew at 22 DAG (d) in *CUC2-GR* plants.

*KNAT2*, and *KNAT6* were slightly decreased, whereas the expression of *STM* was not significantly changed (Figure 4b–d) confirming a rather indirect correlation of high *CUC2* activity and ectopic *STM* expression such as in *CUC2g-m4* leaves. *KNOXI* are widely repressed in leaves, through the activities of transcription factors such as AS1, AS2, SAW1, and SAW2.[6,7,9,10] Nevertheless, we found increased levels of all four *KNOXI* genes in *cuc2-3* mutants (Figure S3A-D) suggesting that *CUC2* could be part of the group of transcription factors, which suppresses *KNOXI* gene expression in wild-type leaves.

Next, we checked an up-stream component of the *CUC2* signaling network controlling leaf serration. *DEVELOPMENT-RELATED PcG TARGET IN THE APEX4* (*DPA4/NGAL3*) negatively regulates *CUC2* expression independently of *MIR164A* preventing strong serration.[39] The mRNA levels of *DPA4* were slightly decreased at 3 HAI of *CUC2-GR* (Figure 4e), whereas *DPA4* was up-regulated in *cuc2-3* mutants (Figure S4) indicating a double-negative feedback loop between *CUC2* and *DPA4*.

In contrast to *CUC1*,[16] *CUC3* is involved in leaf serration.[40] Genetic analyses showed that *CUC2* promotes leaf serration via two different pathways, one early independent of *CUC3* promoting teeth emergence and outgrowth, and one latter requiring both *CUC2* and *CUC3*, which sustain teeth formation.[40] Although this genetic interactions and similar expression domains suggest *CUC2* might be upstream of *CUC3*,[40] we did not find any significant changes of *CUC3* in response to temporary induced *CUC2-GR* (Figure 4f).

DEX-treated *CUC2-GR* leaves (Figures 2 and 3, S2) phenocopied either the smaller leaves of *er* mutants [Figure S5][28,29,41] or the much smaller leaves of *er erl1 erl2* triple mutants.[30] The petiole length of continuously induced *CUC2-GR* can be dramatically reduced to a certain degree of almost distinction (Figures 2f,g and 3c) that remarkably resembles the leaf phenotype of *er erl1 erl2* triple mutants that lack petiole elongation.[30] However, in average the petiole length of continuously induced *CUC2-GR* plants was reduced to 40.7% (Figure S3B) that reminds of the shorter petiole in L*er* plants (Figure S5D). *EPFL2* encodes one of the ligands of the ER-like receptor kinases ER ERL1 and ERL2.[26] Three hours after DEX induction of *CUC2-GR* plants, *EPFL2* mRNA levels had been significantly reduced compared with the non-DEX treated plants (Figure 4g), whereas *EPFL2* was up-regulated in the *cuc2-3* loss-of-function mutants and might be slightly reduced in *cuc2-1D* mutants (Figures 5 and 6a). *cuc2-1D epfl2* mutants have teeth in mature leaves even if the teeth are smaller than those of *cuc2-1D* indicating that *CUC2* can promote tooth growth in an *EPFL2*-independent manner.[26] Nevertheless, the reduction of the tooth size of *cuc2-1D* leaves by loss of *EPFL2* function suggests that the ectopic tooth growth is partially

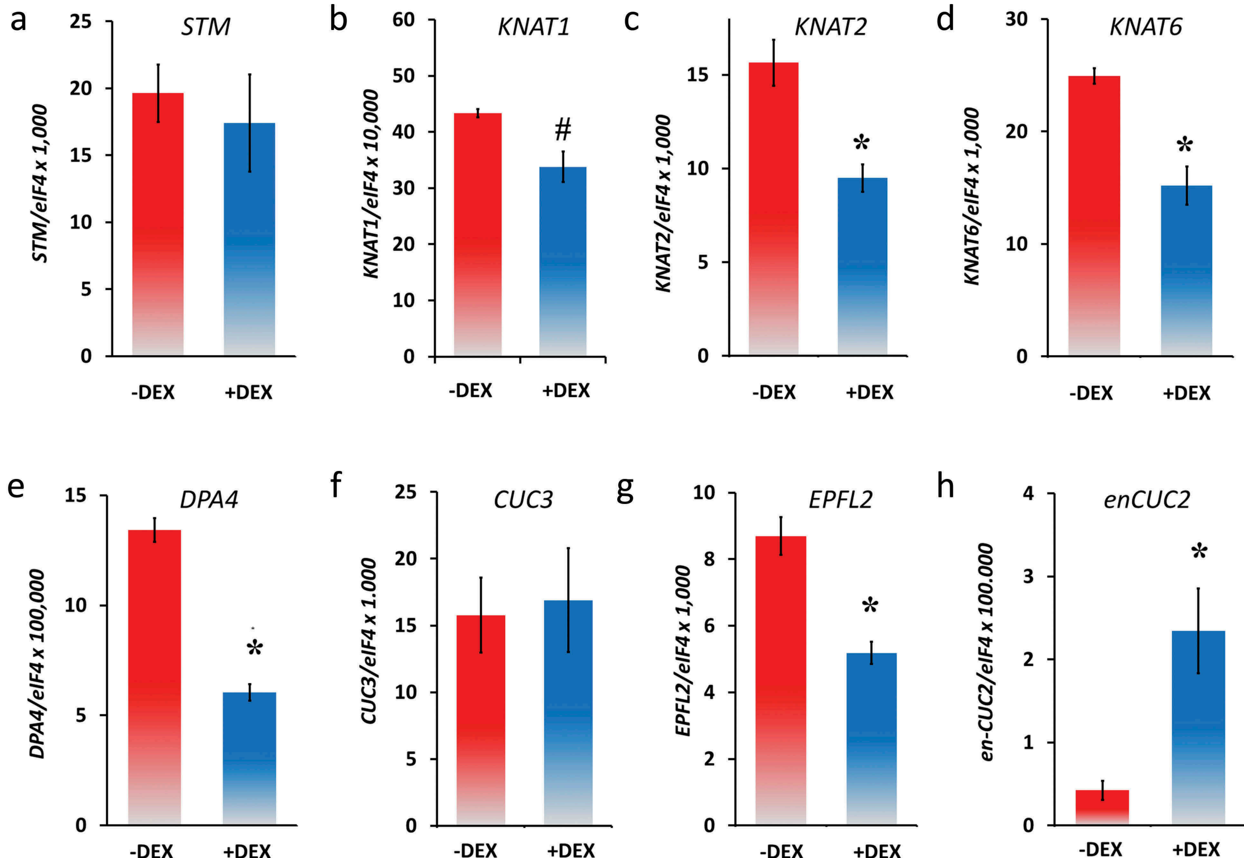


**Figure 4.** Expression analysis of genes related to leaf development in none and DEX-induced *CUC2-GR* leaves (qRT-PCR, N = 3, ± SE), 26 DAG, 3 HAI. A, *STM*, B, *KNAT1* (*BP*), C, *KNAT2*, D, *KNAT6*, E, *DPA4*, F, *CUC3*, G, *EPFL2* and H, endogenous *CUC2* (*en-CUC2*). Asterisks indicate a significant change of expression (Student's t-test: *, $P < .05$; #, $P = .07$) compared with the non-treated control.

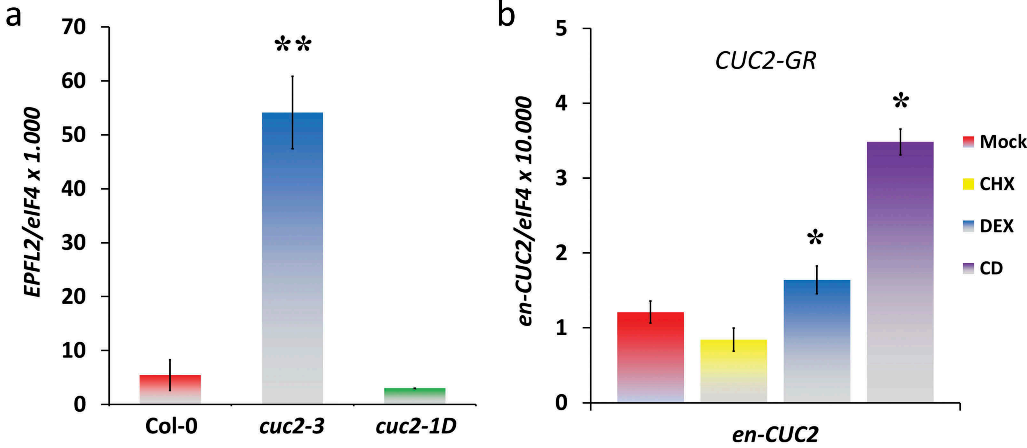


**Figure 5.** Expression analysis of *EPFL2* and endogenous *CUC2* (*en-CUC2*) in seedlings (qRT-PCR, N = 3, ± SE). A, Col-0, *cuc2-3* and *cuc2-1D* seedlings, 26 DAG. B, *CUC2-GR* seedlings were mock-treated (mock) or treated with cycloheximide (CHX), dexamethasone (DEX), or dexamethasone plus cycloheximide (CD). Asterisks indicate significant change of expression (Student's t-test: *, $P < .05$; **, $P < .01$) compared with Col-0 or mock-treated *CUC2-GR* plants, respectively.

dependent of EPFL2-ER ligand-receptor module. Alternatively, ectopic *CUC2* promotes serration also by cell autonomous repression of both growth and *EPFL2* expression in the sinus and other areas of the leaf plate.

In order to test the response of the *CUC2* promoter to transient higher *CUC2* levels, we designed gene-specific amplification primers derived from untranslated region sequences that specifically amplified the endogenous *CUC2* (*en-CUC2*) but not the transgenic *CUC2-GR* mRNA. Interestingly, *en-CUC2* was significantly induced by *CUC2-GR* in the qRT-PCR analysis 3 HAI with DEX (Figure 4h). That indicates that *CUC2* could induce its

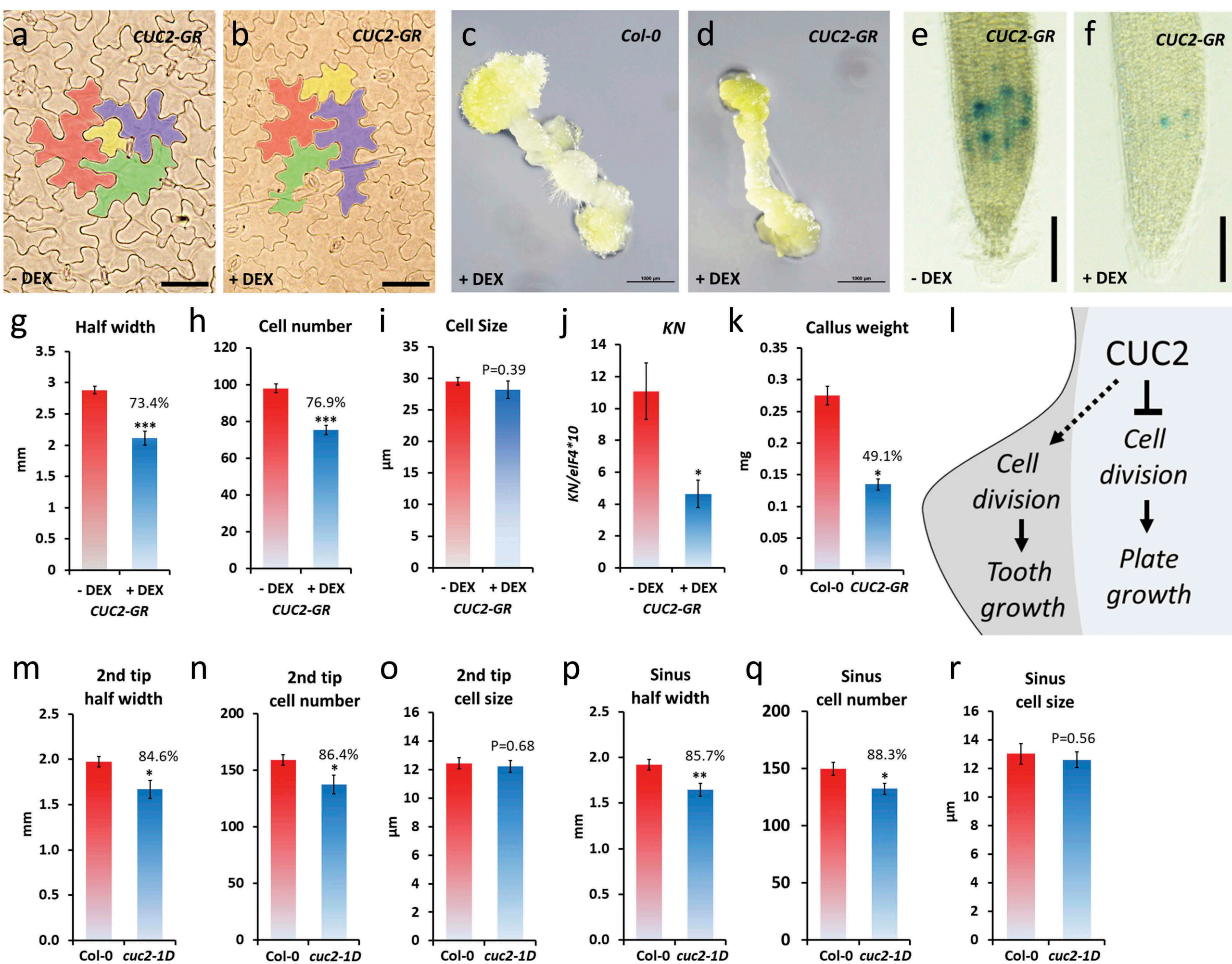


**Figure 6.** Growth repression by ectopic *CUC2* based on reduced cell numbers. A-K, continuously DEX-treated *CUC2-GR* plants (+ DEX) in comparison to non-treated (-DEX) or Col-0. A-B, pavement epidermis cells in the fourth leave of *CUC2-GR* plants, 30 DAG, four cells of representative cell size range were marked by false colors. C-D, Callus of hypocotyl explants of DEX-treated *CUC2-GR* and Col-0 plants, 16 days on callus-inducing media (CIM). E-F, *CYCB1;1::GUS* expression in *CUC2-GR* roots. G-I, Half leaf width, cell number and cell size (diameter) was measured from the midrib to the tooth tip of the fourth leaf, 30 DAG, N ≥ 10, ± SE. J, expression of the cell cycle marker *KN* in *CUC2-GR* seedlings, 7 DAG (qRT-PCR, N = 3, ± SE), 7 DAG. E, Callus weight of hypocotyl explants of DEX-treated *CUC2-GR* and Col-0 plants, after 7 weeks on CIM, N = 20, ± SE. L, Conceptual model of leaf growth control by *CUC2*. M-R, Fourth leaf of Col-0 and *cuc2-1D* plants. Half leaf width, cell number and cell size (diameter) were measured from the midrib to the 2$^{nd}$ tooth tip (2$^{nd}$ tip) or the sinus between 1$^{st}$ and 2$^{nd}$ tooth (sinus), respectively, 20 DAG long day, N = 10, ± SE. Asterisks indicate significant differences (Student's t-test: *, $P < .05$; **, $p < .01$) compared with the controls.

own expression by direct transcriptional activation or by binding to fast-reacting target genes encoding transcription factors, which activate *CUC2* in turn. Nevertheless, as the transcription factor DPA4 is meant to be an up-stream repressor of *CUC2*,[39] the repression of *DPA4* could also play a role in indirect up-regulation of *en-CUC2* in induced *CUC2-GR* plants. To test this hypotheses, we used cycloheximide (CHX) as a translation inhibitor[42] and found even higher *en-CUC2* expression in the samples treated with both, DEX and CHX suggesting that the activation of *en-CUC2* by CUC2-GR is rather directly (Figure 5b).

To summarize, we found that some key regulators of leaf development, supposed to be genetically downstream of *CUC2*, do not react to temporary increased *CUC2*, such as *CUC3* and *STM*, or are surprisingly downregulated such as *KNAT1,2,6*, *DPA4*, and *EPFL2*.

### 2.5. Ectopic CUC2 limits growth rather by cell cycle inhibition than by cell size control

Previous studies about the role of *CUC2* in leaf serration focused on either promotion of tooth growth or growth repression in the sinus.[14,16,22,23,40,43] *CUC2* contributes to the outgrowth of leaf teeth by promoting non-cell autonomously cell proliferation.[22] Nevertheless, this does not answer how the cell autonomous function of *CUC2* causes the growth inhibition in the leaf sinus of wild-type or plants with ectopic *CUC2* expression. To determine the effects of ectopic *CUC2* expression on cell size and cell number, we cleared rosette leaves of continuously induced *CUC2-GR* and control plants, 30 DAG. Like in the epidermis of wild-type leaves, the size of pavement cells varies strongly in *CUC2-GR* but we did not find any obvious changes in the overall cell size between induced and non-induced plants (Figure 6a,b). As a more

direct test of the hypothesis that *CUC2* controls rather numbers of cell than cell size, we examined the distance (half leaf width), cell number and cell diameters along a line between midrib to the tooth tip of the cleared leaves (Figure 6g–i). We found a strong correlation between the reduction of the half leaf wide, and the cell number (26.6% or 23.1%, respectively; Figure 6g–h) in continuously induced *CUC2-GR* indicating that ectopic *CUC2* inhibits cell proliferation (Figure 6l). To test this hypothesis, we measured the expression of *KNOLLE* (*KN*), which encodes an M phase-specific syntaxin, involved in vesicle fusion during cytokinesis,[44] by qRT-PCR. In continuously induced *CUC2-GR* seedlings, the abundance of *KN* transcripts was reduced more than half in comparison to the control (Figure 6j).

In order to test the general capacity of ectopic *CUC2* in cell cycle inhibition, we examined the effects in callus and roots. Continuously induction of *CUC2-GR* significant reduced callus size and weight on callus inducing media (CIM) (Figure 6c–d,k). In continuously induced *CUC2-GR* root tips, expression of the cell cycle marker *CYCB1;1::GUS* was strongly reduced (Figure 6e–f, S6) suggesting that ectopic *CUC2* can partially inhibit cell cycle in various tissues.

Next, we examined the half leaf width, cell number, and cell diameters also in *cuc2-1D* leaves (Figure 6m–r). As shown before, we found slightly deeper serration in the sinus between the first and second tooth (14.3%), which correlated with the slightly reduced cell number (11.7%) between midrib and sinus (Figure 6p–q). Interestingly, also the distance, and accordantly the cell number, between the second tooth tip and midrib was significant reduced (15.3% and 13.6%, respectively; Figure 6m–n). Hence, we did not find any significant changes in the average size of pavement cells in *cuc2-1D* leaves (Figure 6o,r).

To summarize, it was reported before that *CUC2* promotes non-cell autonomously cell proliferation in tooth formation that is even enhanced by ectopic *CUC2* expression.[14,17,22] Here we show that ectopic *CUC2* can repress cell division but did not alter the average epidermis cell size in *CUC2-GR* or *cuc2-1D* leaves suggesting that the cell autonomous growth inhibition by *CUC2* in the sinus and other regions of the leaf plate is mainly caused by reduced cell proliferation.

## 3. Discussion

Leaf shape is controlled by a combination of factors either promote or inhibit growth.[2,45] The transcription factor CUC2 shapes the leaf margin by both growth inhibition in the sinus and promoting non-cell autonomously tooth outgrowth, which involves the growth-promoting phytohormone auxin.[14,23,2,16],developed a computer model of leaf serration basing on the interactions between auxin transport, PIN1 location, and *CUC2* expression during leaf margin development. In the simulation, auxin locally promotes and *CUC2* locally inhibits the propagation of a single cell layer representing the leaf margin.[14] The computer model can successfully simulate the consequences of loss of *CUC2* or *PIN1*, which both cause smooth leaf margins in the simulation as well as in planta, whereas simulating increased *CUC2* expression in the leaf margin produces narrower leaves with deeper serrations.[14] The latter-simulated morphology comes very close to phenotype of *cuc2-1D* and ubiquitous expressed *CUC2-GR* but *cuc2-1D* leaves significant wider and *CUC2-GR* leaves are rather shorter than only narrower (Figure S3A, C). In addition, the computer model does not include the control of cell division in the inner leaf plate,[46] which also influences leaf shape and size. This limitation does not affect the simulation of wild-type leaves, because *CUC2* expression is here limited to the sinus leaf margin, but it matters in leaves with ectopic *CUC2* expression.

Our results suggest that dependent of the spatiotemporal pattern, ectopic *CUC2* expression can enhance either tooth outgrowth or growth inhibition or both. Furthermore, ectopic *CUC2* can repress cell division in the leaf plate causing reduced growth independently whether the plate area is underneath of sinuses or teeth. Temporary induction of *CUC2* represses the *KNOX1* genes *KNAT1, KNAT2* and *KNAT6* (Figure 4b–d). In *as1* mutants, ectopic expression of *KNAT1, KNAT2* and *KNAT6* cause overproliferation in epidermal cells of the leaf petioles which is suppressed in *as1 knat1 knat2 knat6* quadruple mutants.[7] Nevertheless, the expression of *KNOX1* genes is widely silenced in wild-type leaves[8,10,47,48] making it unlikely that cell cycle inhibition by ectopic *CUC2* is widely dependent on further downregulation of *KNAT1, KNAT2*, and *KNAT6*.

Our simplified model in Figure 6l suggests that the sinus area belongs rather to the leaf plate, in which *CUC2* expression can inhibit cell autonomously cell division. As in wild-type leaves *CUC2* expression is limited to a few cells in the sinus,[16] the growth repression is rather weak. Loss of *CUC2* function prevents tooth growth as well as growth inhibition in the sinus but both processes balanced each other so that wild-type and *cuc2-3* leaves have the same total leaf area (Figure 1d). In leaves with ubiquitous expressed *CUC2*, growth repression can affect the whole leave. Furthermore, higher *CUC2* activity, such as in *cuc2-1D*, promotes non-cell autonomously cell division during the ectopic tooth outgrowth (Figure 6l), which depends on the negative feedback loop of auxin and *CUC2* expression.[14]

The increased leaf size in *cuc2-1D* dependents on increased cell proliferation, mediated through an ER-dependent pathway.[17] Interestingly, transient induced *CUC2* can repress *EPFL2* (Figure 4g), which encodes a ligand of the receptors of the ER family promoting cell division in leaves and in general.[26,30,31]*CUC2* can promote growth in leaf teeth both, dependently and independently of *EPFL2*,[26] but repression of *EPFL2* by *CUC2* could still reduce cell proliferation in the leaf plate. Although *EPFL2* is expressed in the whole leaf blade with the exception of vascular and tooth tips, loss of *EPFL2* causes clearly less severe leave size reduction[26] than ubiquitous expressed *CUC2*. This indicates that the repression of *EPFL2* does not play more than a minor role in cell cycle repression by *CUC2*.

It will be a challenge to dissect both *CUC2*-dependent pathways, either promoting cell division in teeth or repressing cell division in the sinus. Our approach, using inducible *CUC2-GR* in time courses, was a first step to understand the developmental timing of both processes. In future studies, temporal induction and reduction of *CUC2* expression under control of the *CUC2* promoter in combination with

separation of the cells of teeth and sinuses will provide more specific and accurate data of the downstream events and might identify different down-stream targets of *CUC2* in both tissues.

## 4. Materials and methods

### 4.1. Plant materials and growth conditions

*Arabidopsis thaliana* (L.) plants were grown at 21°C under short day (10 h light/14 h dark) conditions. Columbia (Col-0, N70000) and Landsberg-0 (La-0, N6765), which were used as wild-type controls, and Landsberg *erecta*-0 (L*er*-0, NW20) and the dominant *cuc2-1D* mutant (N16485) plant lines were obtained from the Nottingham Arabidopsis Stock Center. *cuc2-3*[16,32] and *35S::CUC2-GR* [*CUC2-GR*][19] were kindly provided by Patrick Laufs and Ben Scheres, respectively. Peter Doerner kindly provided seeds of *CYCB1;1::GUS*.[49] With the exception of La-0 and L*er*-0, all genotypes were in Col-0 background. Surface-sterilized seeds were sowed on half-strength Murashige and Skoog salt mix (½ MS; Tian Da Chemical, China) media plates under aseptic conditions. After 10 days, the plants, which had approximately the same size, were transferred to soil in pots.

### 4.2. Dexamethasone treatments

Dexamethasone (DEX) (WAKO, Japan) 10 mmol$L^{-1}$ was dissolved in 70% ethanol and kept at −20°C as a stock solution. In depletion and continues DEX-treatment experiments, *CUC2-GR* plants and Col-0 controls were grown on ½ MS tissue culture plates containing 10 μmol$L^{1}$ (10 μM) DEX for 10 days and then transferred to soil. Plants, growing on soil, were directly sprayed one time per day with a 10 μM DEX solution containing 0.02% Silwet. The *CUC2-GR* and Col-0 seedlings were DEX treated and non-treated either continuously or with specific time courses, starting continuous treatment or withdrawn to different time points, as shown in Figure 2a. Hence, the phenotype of seedlings and dissected leaves were documented only at the last time point.

### 4.3. Phenotypic characterization and microscopy

The seedlings were photographed with a Nikon digital camera (D3200, AF-S Micro NIKKOR 60 mm1:2.8 G ED) in a different time course. The leaves were dissected at specific time points and scanned with a scanner (LIDE220, Cannon, Japan). The smaller leaves image was taken under an SMZ25 Microscope (Nikon, Japan). Leaf silhouettes were generated from scanned leaves by using the following steps in the computer program Photoshop™ (Acrobat Systems incorporated): (I) Extraction of the black background by the magic wand tool (Tolerance, 50), (II) Removing overhanging trichomes and interfering artifacts, (III) Gaussian Blur (Radius, 2.0 pixels), (IV) Unsharp Mask (Amount, 250%; Radius, 500 pixels; Tolerance, 0 levels), (V) Magic wand tool (Tolerance, 50) and Rotate (Transform) for final arrangement of the leaves. All phenotype data were analyzed by using Student's t-test (Excel, Microsoft).

### 4.4. Expression analysis

For RNA extraction, whole seedlings (without roots) or leaves ($1^{st}$ to the $4^{th}$) were collected and snap frozen in liquid nitrogen. The total RNA was purified using TRIZOL (Invitrogen) procedure. The extracted RNA was treated with DNase (Thermo Scientific) to remove DNA contamination. cDNA was synthesized using first-strand cDNA synthesis kit (Thermo Scientific) analyzed by qRT-PCR using the SYBER green supermix (Roche) at the Roche Lightcycler480 II. The DEX plus cycloheximide experiment was described before.[42] The primers used in the qRT-PCR analysis are listed in Table S1.

### 4.5. GUS histochemical assay

*CUC2-GR* ♀ was crossed with *CYCB1;1::GUS* ♂.[49] In the F1 generation, seedlings were grown on ½ MS tissue culture plates containing 10 μmolL-1 DEX or no DEX for 8 days. Detection of GUS activity in the roots was performed with whole seedlings as described with minor modifications.[50]

### 4.6. Callus induction

Col-0 and *CUC2-GR* seeds were sterilized with 70% ethanol (3 times) and germinated on half-strength MS medium (2.165 g/L MS basal medium with vitamin powder, 10 g/L sucrose, and 9 g/L agar, pH 5.8) for 7 days in short-day condition (3 days on fridge before transfer to short day). Hypocotyls were excised and transferred to callus inducing medium (CIM) (4.4 g/L MS basal medium, 10 g/L sucrose, and 9 g/L agar, 2.2 μM 2,4-D, 0.2 μM kinetin, pH 5.8) with 10 μM DEX. Callus weight was measured at 7 weeks after callus induction. Two independent biological replicates were performed.

### 4.7. Tissue clearing and cell counting

For cell counting and visualizing of cell size, rosette leaves were mounted in clearing solution (chloral hydrate:glycerol:water, 8:1:2 [w/w/v]) as described with minor modifications.[52] The measurement of half leaf wide and cell number followed a line between midrib and leaf margin of tooth tip or sinus. The measurement lines ran at a right angle to the midrib. The average cell diameters were calculated by half leaf width/cell number (μm/N).

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## Disclosure of potential conflicts of interest

The authors declare that they have no conflict of interest.

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## ORCID

Xiaoyu Li http://orcid.org/0000-0001-7544-8932
Yucai Zheng http://orcid.org/0000-0003-2893-1803
Qian Xing http://orcid.org/0000-0002-0229-9101
Rhomi Ardiansyah http://orcid.org/0000-0001-9202-8382
Hui Zhou http://orcid.org/0000-0002-1320-9351
Shahid Ali http://orcid.org/0000-0001-6517-320X
Tingting Jing http://orcid.org/0000-0003-2900-0859
Jingjing Tian http://orcid.org/0000-0003-0821-7864
Xing Shun Song http://orcid.org/0000-0001-8129-2612
Yuhua Li http://orcid.org/0000-0002-0405-2823
Ralf Müller-Xing http://orcid.org/0000-0003-0024-4243

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