

F-Box Protein FBX92 Affects Leaf Size in *Arabidopsis thaliana*

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F-box proteins are part of one of the largest families of regulatory proteins that play important roles in protein degradation. In plants, F-box proteins are functionally very diverse, and only a small subset has been characterized in detail. Here, we identified a novel F-box protein FBX92 as a repressor of leaf growth in Arabidopsis. Overexpression of AtFBX92 resulted in plants with smaller leaves than the wild type, whereas plants with reduced levels of AtFBX92 showed, in contrast, increased leaf growth by stimulating cell proliferation. Detailed cellular analysis suggested that AtFBX92 specifically affects the rate of cell division during early leaf development. This is supported by the increased expression levels of several cell cycle genes in plants with reduced AtFBX92 levels. Surprisingly, overexpression of the maize homologous gene ZmFBX92 in maize had no effect on plant growth, whereas ectopic expression in Arabidopsis increased leaf growth. Expression of a truncated form of AtFBX92 showed that the contrasting effects of ZmFBX92 and AtFBX92 gain of function in Arabidopsis are due to the absence of the F-box-associated domain in the ZmFBX92 gene. Our work reveals an additional player in the complex network that determines leaf size and lays the foundation for identifying putative substrates.

Keywords: Cell cycle • F-box protein • Leaf development.

Abbreviations: amiRNA, artificial microRNA; APC/C, anaphase-promoting complex/cyclosome; BB, BIG BROTHER; pBdEF1 α , *Brachypodium distachyon* elongation factor1 α promoter; CaMV, *Cauliflower mosaic virus*; CDC27a, CELL DIVISION CYCLE PROTEIN 27 HOMOLOG A; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; DAS, days after stratification; ICK/KRP, ICK/KIP/CIP-RELATED PROTEIN; FBL17, F-BOX-LIKE17; GFP, green fluorescent protein; GUS, β -glucuronidase; KRP, KIP-RELATED PROTEIN; PRA, projected leaf area; qRT-PCR, quantitative reverse transcription-PCR; SAM, shoot apical meristem; SIM, SIAMESE; SMR, SIM-related; TCP, TEOSINTE BRANCHED1/CYCLOIDEA/PCF; WT, wild type.

Introduction

The leaf is the major photosynthetic organ of plants, and as such provides the basis for food, feed and bio-energy production by humans. Because leaf size, shape and number strongly

influence photosynthetic capacity, understanding the molecular networks underneath is pivotal for future food security. The largest part of leaf development occurs post-embryonically: leaf primordia are initiated at the flanks of the shoot apical meristem in a position that is characteristic for the species. During a first phase after leaf initiation, cells are only proliferating because cell division and expansion are balanced (Donnelly et al. 1999, Andriankaja et al. 2012, Gonzalez et al. 2012), which is followed by a phase of cell expansion starting at the tip of the leaf, thereby establishing a cell cycle arrest front that remains in both *Arabidopsis* and maize at a constant position for a few days, and then rapidly declines (Andriankaja et al. 2012, Avramova et al. 2015). From then on, leaves enlarge solely because of an increase in average cell size due to cell expansion and because of meristemoid divisions, generating extra pavement cells while forming stomata, until the leaf reaches its final size (Gonzalez et al. 2012). Both leaf initiation and its further growth are under control of a complex set of intrinsic signals, such as phytohormones, that regulate cell proliferation and cell expansion to elaborate the final shape and size of the leaf (Gonzalez et al. 2012, Powell and Lenhard 2012, González and Inzé 2015).

The cell division cycle is precisely controlled to duplicate the DNA correctly during the S-phase and to generate two daughter cells during mitosis. Complexes formed by cyclin-dependent kinase (CDK) and cyclin are the core cell cycle regulators that play crucial roles at both the G₁ to S and the G₂ to M phase transitions (Harashima et al. 2013). The activity of these complexes is regulated by (de)phosphorylation, interaction with inhibitors and targeted protein degradation (De Veylder et al. 2007). Targeted protein degradation of cell cycle regulators happens through the ubiquitin–proteasome pathway, a highly precise post-translational regulatory process that ensures irreversibility of cell cycle progression (Genschik et al. 2014). The ubiquitin–proteasome pathway involves covalent attachment of polyubiquitin chains to targeted substrate proteins through the consecutive action of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) (Hotton and Callis 2008). Ubiquitinated proteins are subsequently recognized and degraded via the 26S proteasome (Hershko and Ciechanover 1998). Substrate specificity of the pathway is defined by the E3 ligase, which binds to specific target proteins and stimulates in this way the conjugation of ubiquitin to this target protein.

In *Arabidopsis thaliana* (*Arabidopsis*), >1,400 genes or approximately 5% of the proteome encode elements of the ubiquitin–proteasome pathway (Smalle and Vierstra 2004). Several mutants and plants transgenic for the ubiquitin–proteasome pathway with altered leaf size have been identified, illustrating that controlled proteolysis is an important layer of regulation during development. For instance, a mutation in the E3 ubiquitin ligases BIG BROTHER (BB or EOD1) and DA2 prolongs the timing of cell proliferation in different organs (Disch et al. 2006, Xia et al. 2013). BB/EOD1 and DA2 act synergistically with the ubiquitin receptor DA1, and inactivation of DA1 causes the formation of larger plants (Li et al. 2008). Similarly, loss of function of a subunit of the 19S proteasome, RTP2A, results in larger organs due to an increased cell size partially compensated by a reduced cell number (Kurepa et al. 2009). Also the PEAPOD transcription factors, negative regulators of meristemoid activity, are subjected to F-box-mediated proteolysis by STERILE APETAL (SAP) (Wang et al. 2016). Another important system that enables ubiquitin-mediated degradation of proteins important for growth is the multiprotein E3 ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C) (De Veylder et al. 2007, Eloy et al. 2015). The subunits APC10 (Eloy et al. 2011) and CELL DIVISION CYCLE PROTEIN 27 HOMOLOG A (CDC27a) (Rojas et al. 2009) both promote cell proliferation and an increased leaf size, whereas SAMBA is a plant-specific negative regulator of the APC/C and its inactivation increases organ size (Eloy et al. 2012).

A major type of E3 ligases that is involved in cell cycle control are the SCF E3 ligases, which consist of four components, Cullin1/Cdc53, Rbx1/Roc1/Hrt1, Skp1 (ASK1 in plants) and an F-box protein (Cardozo and Pagano 2004). The scaffold protein Cullin1 interacts at its C-terminus with Rbx1/Roc1/Hrt1, which binds to the E2 loaded with ubiquitin, and at its N-terminus with Skp1, which binds to the F-box protein that interacts with the target proteins for degradation (Bai et al. 1996). The SCF complex has an important function in the proteolysis of cell cycle regulatory proteins, although it can also mark other proteins for destruction (Genschik et al. 2014). More precisely, the SCF complex plays a critical role during the G₁ to S phase transition, which requires the degradation of CDK inhibitors (CKIs, also known as ICK/KRP proteins) to release CDK activity (Verkest et al. 2005, Ren et al. 2008, Noir et al. 2015). For example, the F-BOX-LIKE17 (FBL17) is essential to maintain normal cell proliferation by mediating the degradation of the CDK inhibitor KIP-RELATED PROTEIN2 (KRP2) known to switch off CDKA₁ kinase activity (Noir et al. 2015).

F-box proteins identify the target proteins for degradation, recruit them and position them in proximity to E2 for ubiquitination (Skaar et al. 2013). Via their F-box domain, a structural motif consisting of approximately 50 conserved amino acids (Xiao and Jang 2000), they are anchored to the SCF complex, whereas the C-terminal domain binds the target proteins for ubiquitination and degradation usually via protein interaction motifs. Examples of these protein interaction motifs occurring in plants are kelch repeats, WFBX920, LRR and tubby (Gagne et al. 2002, Kuroda et al. 2002, Jain et al. 2007, Jia et al. 2013). F-box proteins in plants belong to a large family: about 700 F-box

proteins have been identified in *Arabidopsis* (Risseuw et al. 2003) and *Oryza sativa* (rice) (Jain et al. 2007) and about 350 in *Zea mays* (maize) (Jia et al. 2013), numbers that are much larger than the number of F-box proteins identified in most other eukaryotes (Gagne et al. 2002, Schumann et al. 2011). They are involved in a large variety of biological processes, including flower development, hormone perception and signaling, circadian rhythms, defense responses, senescence, embryogenesis and seedling development (Lechner et al. 2006, Hua et al. 2011, Schumann et al. 2011). Evidence is coming to light that E3 ligases might act as receptors of hormones or other molecules for signal transduction, suggesting a novel mechanism to link internal and external cues to cell division (Jurado et al. 2008, Achard and Genschik 2009, del Pozo and Manzano 2014). However, the specific function of the majority of the F-box proteins currently remains unclear (Schumann et al. 2011).

Here, we identified a maize F-box protein, ZmFBX92, for which ectopic expression in *Arabidopsis* resulted in plants with larger leaves, although maize plants overexpressing ZmFBX92 showed no obvious phenotypes. In contrast, gain-of-function mutants of the *Arabidopsis* FBX92 homologous gene, AtFBX92, developed smaller leaves than the wild type (WT) due to a reduced cell number. In concert, *Arabidopsis* plants with reduced AtFBX92 expression levels had larger leaves due to an increased cell division rate and consequential cell number. The opposite effects of ZmFBX92 and AtFBX92 gain of function in *Arabidopsis* can be explained by the presence of an F-box-associated domain in the AtFBX92 gene that is lacking in the ZmFBX92 gene. Overexpression of a truncated AtFBX92 allele lacking the F-box-associated domain resulted in a larger leaf size phenotype than in plants with reduced levels of AtFBX92 or which ectopically expressed ZmFBX92. In addition, the increase in expression levels of several cell cycle genes in plants with reduced levels of AtFBX92 indicates that this newly identified F-box protein AtFBX92 acts as a repressor of leaf growth by affecting cell division.

Results

Ectopic ZmFBX92 expression in *Arabidopsis* positively affects leaf size

In the maize genome, about 350 F-box proteins have been identified and a small subset has previously been reported to respond to multiple stress treatments, such as salt, drought, cold and heat (Kakumanu et al. 2012, Jia et al. 2013). We investigated whether expression of one of these F-box genes, ZmFBX92, in *Arabidopsis* would alter plant growth under mild osmotic stress. Therefore, ZmFBX92 was expressed in *Arabidopsis* under control of the constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter and five independent, homozygous, single-locus lines were selected to analyze their phenotype. The results were very similar for all lines, thus only the results of line ZmFBX92^{OE}11, hereafter ZmFBX92^{OE}, are presented.

Phenotypic analysis of ZmFBX92^{OE} and WT [Columbia-0 (Col-0)] plants revealed that the leaf area was significantly

larger in the transgenic plants, while no other phenotypic abnormalities were observed (Fig. 1; Supplementary Fig. S1A). The projected rosette areas (PRAs) of WT and *ZmFBX92^{OE}* plants grown in vitro in standard and mild osmotic stress conditions (25 mM mannitol) were determined from 6 until 21 days after stratification (DAS) (Fig. 1A). On average, osmotic stress reduced the rosette area by about 60% at 21 DAS. *ZmFBX92^{OE}* plants had a significantly increased rosette area compared with the WT under both conditions (Fig. 1A). At 21 DAS, this difference was about 35% and about 50% under control and mild stress conditions, respectively. The increased rosette area in *ZmFBX92^{OE}* occurred already very early during development: at 6 DAS the rosette size was about 50% larger in transgenic plants than in the WT under both control and mild stress conditions (Fig. 1A, inset). There was no significant interaction effect ['three-way' analysis of variance (ANOVA)], indicating that the effect of *ZmFBX92^{OE}* expression on plant growth was comparable under both control and mild osmotic stress conditions. Hence, for further analysis, we focused on rosette growth and leaf development under control conditions.

The positive effect of *ZmFBX92^{OE}* on leaf size in Arabidopsis was corroborated by determining the individual leaf areas at 22 DAS (Fig. 1B, C). Both mature and juvenile leaves were larger in *ZmFBX92^{OE}* plants. To examine to what extent a difference in cell proliferation and/or cell expansion was responsible for the increased leaf size, the number and size of abaxial epidermal cells were compared in WT and *ZmFBX92^{OE}* leaves. The fully mature (22 DAS) third leaf was approximately 30% larger in *ZmFBX92^{OE}* plants due to a highly increased cell number (approximately 70%), which was partially compensated by a reduction in cell size of approximately 20% (Fig. 1D). Thus, ectopic expression of *ZmFBX92* in Arabidopsis resulted in larger leaves primarily due to an increased cell number.

ZmFBX92 overexpression in maize has no effect on leaf size

To investigate if variation in *ZmFBX92* expression levels also affects plant development in maize, we generated three independent, single-locus lines overexpressing *ZmFBX92* under control of the *Brachypodium distachyon* elongation factor1 α promoter (pBdEF1 α) (Coussens et al. 2012), showing an approximately 50- to 800-fold higher expression level than the control inbred line B104 (Supplementary Fig. S2A). Leaf growth under control and mild drought conditions was monitored in the two lines with the highest overexpression level, *ZmFBX92^{OE1}* and *ZmFBX92^{OE2}*. Several final leaf size-related parameters were determined, i.e. area, width, length and weight, and these measurements were complemented with the kinetic parameters 'leaf elongation rate' and 'leaf elongation duration' (Voorend et al. 2014) and shoot-related parameters at seedling stage, i.e. fresh weight, V-stage and leaf number (Supplementary Fig. S2B–K) (Baute et al. 2015). Mild drought stress negatively impacted all these parameters, except leaf elongation duration, which was prolonged (Supplementary Fig. S2D), implying that mild drought stress primarily reduces growth rate. When comparing growth of *ZmFBX92^{OE}* and

control B104 plants, no significant differences were observed, for any of the measured parameters, under control conditions or under mild drought stress (Supplementary Fig. S2B–K).

Altered AtFBX92 expression level influences leaf size by affecting cell number

The putative orthologous gene of *ZmFBX92* in Arabidopsis was identified using PLAZA (Proost et al. 2015), and is further designated *AtFBX92* (At3g07870). To examine the function of this gene in Arabidopsis, we generated plants with altered *AtFBX92* expression levels. Transgenic Arabidopsis plants were generated that ectopically overexpressed *AtFBX92* under the control of the constitutive CaMV 35S promoter. Four independent transformation events with a single-insertion locus and varying expression levels of *AtFBX92* were selected for further analysis (Supplementary Fig. S1B). Because the phenotypes of the lines with the weakest overexpression were similar, we present here only the analysis of *AtFBX92^{OE7}* together with that of *AtFBX92^{OE2}* showing a stronger increase in expression level.

In contrast to what was observed for plants that ectopically expressed *ZmFBX92*, *AtFBX92^{OE}* plants showed a reduction in the rosette area compared with WT plants (Fig. 2A; Supplementary Fig. S1C). This reduction was comparable under mild osmotic stress (Supplementary Fig. S3A). No other obvious phenotypes were observed in *AtFBX92^{OE}* plants. The decreased rosette size was visible already very early during development (Fig. 2A, inset), from 6 DAS onwards. The negative effect of *AtFBX92* overexpression on leaf growth was confirmed by determining the individual leaf areas of 22-day-old plants grown in vitro. The areas of the mature leaves were significantly smaller in the *AtFBX92^{OE7}* plants compared with the WT, whereas for *AtFBX92^{OE2}* plants, all leaves were significantly smaller, including the younger leaves (Fig. 2B, C). To explore the cellular basis of the leaf size decrease, leaf development of *AtFBX92^{OE}* and WT plants grown in vitro was analyzed at the cellular level. Similar to the case for *ZmFBX92^{OE}*, cell number and cell size of the abaxial epidermis of the third leaf were determined at 21 DAS, when this leaf is fully matured (Fig. 2D). The mature third leaf of *AtFBX92^{OE2}* and *AtFBX92^{OE7}* was 45% and 16%, respectively, smaller than that of the WT, due to a strong reduction in cell number (52% and 22% for *AtFBX92^{OE2}* and *AtFBX92^{OE7}*, respectively), which was partially compensated by an increased cell size (15% and 7% for *AtFBX92^{OE2}* and *AtFBX92^{OE7}*, respectively).

In parallel with the plants overexpressing *AtFBX92*, transgenic plants with reduced expression levels were generated by designing an artificial microRNA (amiRNA) targeting *AtFBX92* using the tool at <http://wmd3.weigelworld.org/cgi-bin/webapp.cgi> (Ossowski et al. 2008). Three homozygous, independent, single-locus lines with reduced expression levels (Supplementary Fig. S1D) were analyzed for their leaf phenotype and, because they were very similar, only the results of *amiFBX92-4*, hereafter designated *amiFBX92*, is presented (Fig. 3; Supplementary Fig. S1E). The PRA was determined from 5 until 21 DAS and shown to be larger in *amiFBX92* compared with the WT from the first day of analysis onwards

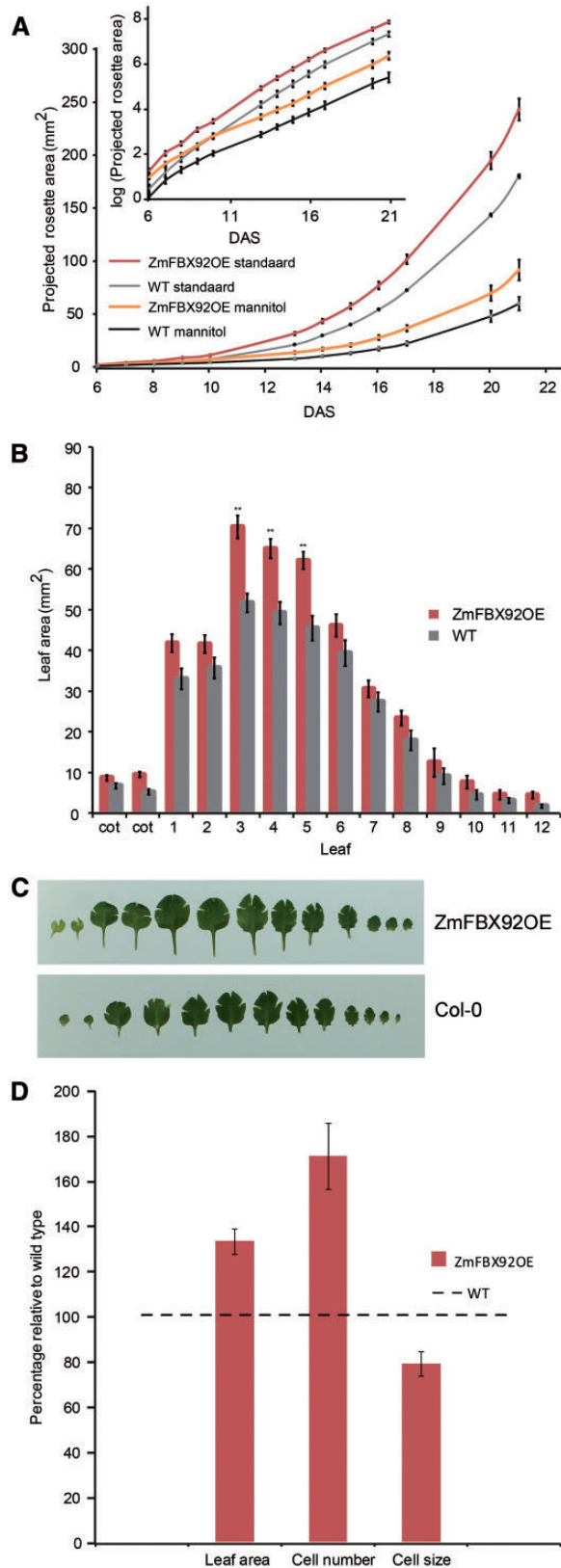


Fig. 1 Effect of *ZmFBX92* expression on rosette and leaf growth in *Arabidopsis* and cellular basis of the leaf size differences. (A) PRA of a *ZmFBX92*^{OE} transgenic line and the WT over time from 6 until 21 DAS. Plants were grown in vitro on standard medium or medium containing 25 mM mannitol. Inset: PRA in log scale. Values represent the mean \pm SE ($n_{\text{transgenic line}} = 25\text{--}27$, $n_{\text{WT}} = 39$). (B) Individual leaf size

(Fig. 3A, inset). This increase in PRA was comparable under mild osmotic stress (Supplementary Fig. S3B). Next, the individual leaf areas were determined at 21 DAS. All leaves except leaf 3 of *amiFBX92* were significantly larger than those of the WT (Fig. 3B, C). Cellular analysis of the fully matured first leaf pair at 21 DAS showed that the increase in leaf area (24%) was due to an increased cell number (47%), partially compensated by a decreased cell size (16%) (Fig. 3D). Taken together, our data indicate that altering *AtFBX92* expression levels influences leaf size in opposing ways, which is primarily the result of a difference in cell number.

Opposing phenotypes in *ZmFBX92*- and *AtFBX92*-overexpressing plants

Unexpectedly, ectopic expression of *ZmFBX92* and overexpression of *AtFBX92* in *Arabidopsis* resulted in opposite leaf phenotypes. A sequence search using Pfam (Finn et al. 2014) and InterPro revealed that *AtFBX92* harbors an F-box-associated domain, type 3, C-terminally of the F-box domain, whereas this F-box-associated domain is lacking in *ZmFBX92* (Supplementary Fig. S4). To analyze if overexpression of *AtFBX92* without the F-box-associated domain has a phenotype comparable with ectopic expression of *ZmFBX92*, a deletion mutant construct *AtFBX92*^{del} was generated, expressing the N-terminal part of the gene including the F-box domain but not the F-box-associated domain, under the control of the constitutive CaMV 35S promoter (Supplementary Fig. S5). Three independent, single-locus lines with high expression levels of *AtFBX92*^{del} (Supplementary Fig. S1F) were selected for further phenotypic characterization. Because the leaf phenotypes of the three lines were very similar in vitro, only the results for *AtFBX92*^{del}12 with the lowest expression level, hereafter named *AtFBX92*^{del}, are shown. Quantitative image analysis of the PRA over time, from 6 DAS until 24 DAS, indicated that *AtFBX92*^{del} plants were larger than the WT (Fig. 4A). Additionally, the PRA increase started very early during development (Fig. 4A, inset), similar to the effect observed in *ZmFBX92*^{OE} and *amiFBX92* lines. In agreement with this, the individual leaf areas determined at 20 DAS were significantly larger than those of the WT (Fig. 4B, C). Consistently, this increase in size of the first leaf pair (27%) at 20 DAS was due to an increased cell number (29%), while there was no significant effect on cell size (Fig. 4D).

AtFBX92 expression pattern

To analyze the spatiotemporal expression pattern of *AtFBX92*, we engineered a construct consisting of the 1.3-kb fragment upstream of the ATG codon of *AtFBX92* fused to a green fluorescent protein (GFP)- β -glucuronidase protein (GUS) reporter

Fig. 1 Continued

of 22-day-old WT and *ZmFBX92*^{OE} plants grown in vitro. Values represent the mean \pm SE ($n = 7$). Significant differences (Student's *t*-test): * $P < 0.05$; ** $P < 0.01$ relative to the WT. (C) Representative pictures from the measurements shown in (B). (D) Average area, pavement cell number and pavement cell size of leaf 3 at 22 DAS of *ZmFBX92*^{OE} plants relative to the WT. Values represent the mean \pm SE ($n = 3$).

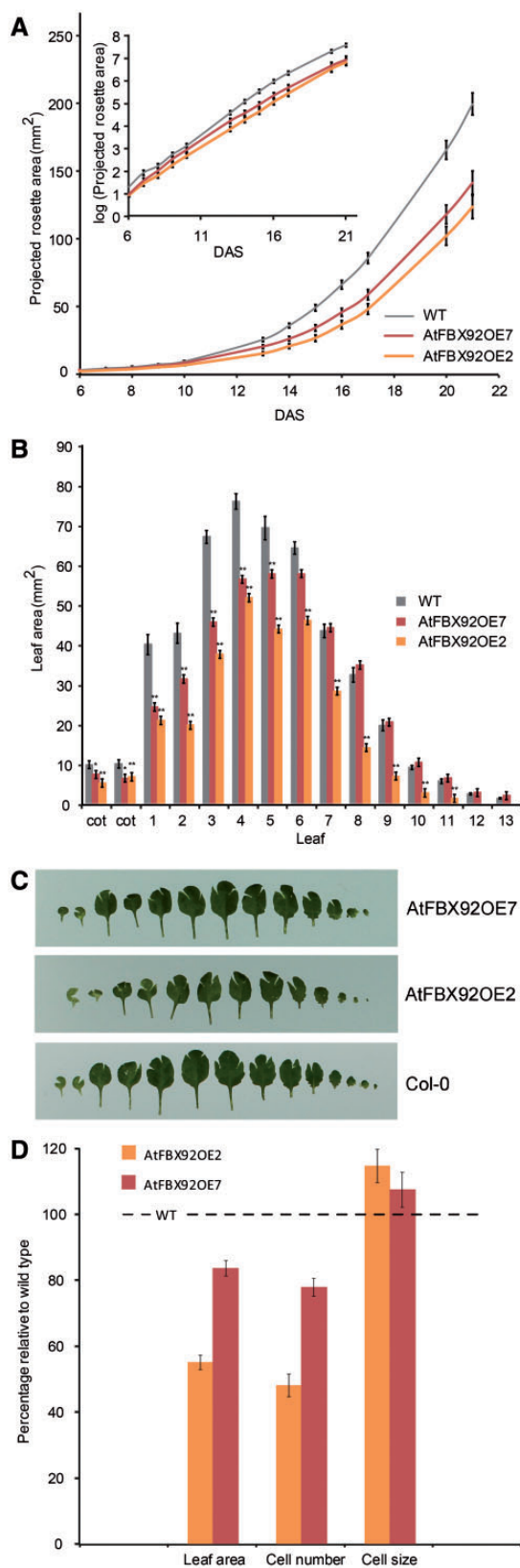


Fig. 2 Effect of *AtFBX92* ectopic expression on rosette and leaf growth under standard conditions in vitro and cellular basis of leaf size differences. (A) PRA of *AtFBX92*^{OE} transgenic lines and the WT over time from 6 until 21 DAS. Plants were grown in vitro on standard medium. Inset: PRA in log scale. Values represent the mean ± SE ($n_{\text{transgenic lines}} = 26\text{--}32$, $n_{\text{WT}} = 36$). (B) Individual leaf size of 22-day-old WT, *AtFBX92*^{OE7}

cassette in a FAST vector (Shimada et al. 2010). This construct, named *pAtFBX92:GFP:GUS*, was subsequently introduced into Arabidopsis plants. Histochemical analyses of three independent Arabidopsis transgenic lines showed a similar expression pattern (Fig. 5). In general, *AtFBX92* was widely expressed in young seedlings, although expression seemed somewhat lower in the hypocotyl (Fig. 5A), and strongly in the whole root, except for the most distal region of the basal meristem (Fig. 5B). *AtFBX92* expression was observed as leaf development progressed from proliferative to mature tissue, although expression was somewhat lower in younger than in older leaves (Fig. 5C). This is in agreement with the expression levels in the transcriptomics data set of leaf 3 during the subsequent phases of proliferation, expansion and maturation, showing that *AtFBX92* expression was low in fully proliferative tissue and increased gradually towards maturity (Supplementary Fig. S6) (Andriankaja et al. 2012). In 6-week-old plants, *GUS* expression was also detected in the stems, sepals, style, the most distal part of carpels and in pollen grains of reproductive tissues (Fig. 5D). *GUS* expression was also detected in developing siliques, in the valves but not in seeds (Fig. 5E).

Rosette growth in soil

To validate the in vitro observed differences in rosette size of plants with altered *AtFBX92* expression levels, we grew *AtFBX92*^{OE}, *amiFBX92*, *AtFBX92*^{del} and the corresponding WT plants in soil on the automated imaging platform WIWAM, which allows the PRA to be followed over time (Skirycz et al. 2011b). Two lines of each construct were analyzed. We could confirm that also in soil, *AtFBX92*^{OE} plants were smaller than the WT, whereas *amiFBX92* and *AtFBX92*^{del} plants were larger, although for *AtFBX92*^{del} plants, this could only be confirmed for one of the two lines (Fig. 6A; Supplementary Fig. S7). In general, the effects in soil seemed to be less pronounced than in vitro, because the absolute percentage difference from the WT was always larger in vitro than in soil. At 20 DAS, for instance, the PRA of *amiFBX92* grown in vitro was 25% larger than that of the WT vs. 15% when grown in soil; 32% for *AtFBX92*^{del} grown in vitro vs. 18% when grown in soil; and −38% and −29% for *AtFBX92*^{OE2} and *AtFBX92*^{OE7}, respectively, grown in vitro vs. −14% and −16%, respectively, when grown in soil. The PRAs of *amiFBX92* and *AtFBX92*^{del} plants grown in soil were significantly larger than those of the WT already very early during development (6 DAS), similar to plants grown in vitro (Supplementary Fig. S7B, C, insets). However, *AtFBX92*^{OE} lines grown in soil were only significantly smaller than the WT from 18 DAS onwards (Supplementary Fig. S7A), whereas in vitro a significant reduction in rosette growth for

Fig. 2 Continued

and *AtFBX92*^{OE2} plants grown in vitro. Values represent the mean ± SE ($n = 7$). Significant differences (Student's *t*-test): * $P < 0.05$; ** $P < 0.01$ relative to the WT. (C) Representative pictures from the measurements shown in (B). (D) Average area, pavement cell number and pavement cell size of leaf 3 at 21 DAS of *AtFBX92*^{OE7} and *AtFBX92*^{OE2} plants relative to the WT. Values represent the mean ± SE ($n = 3$).

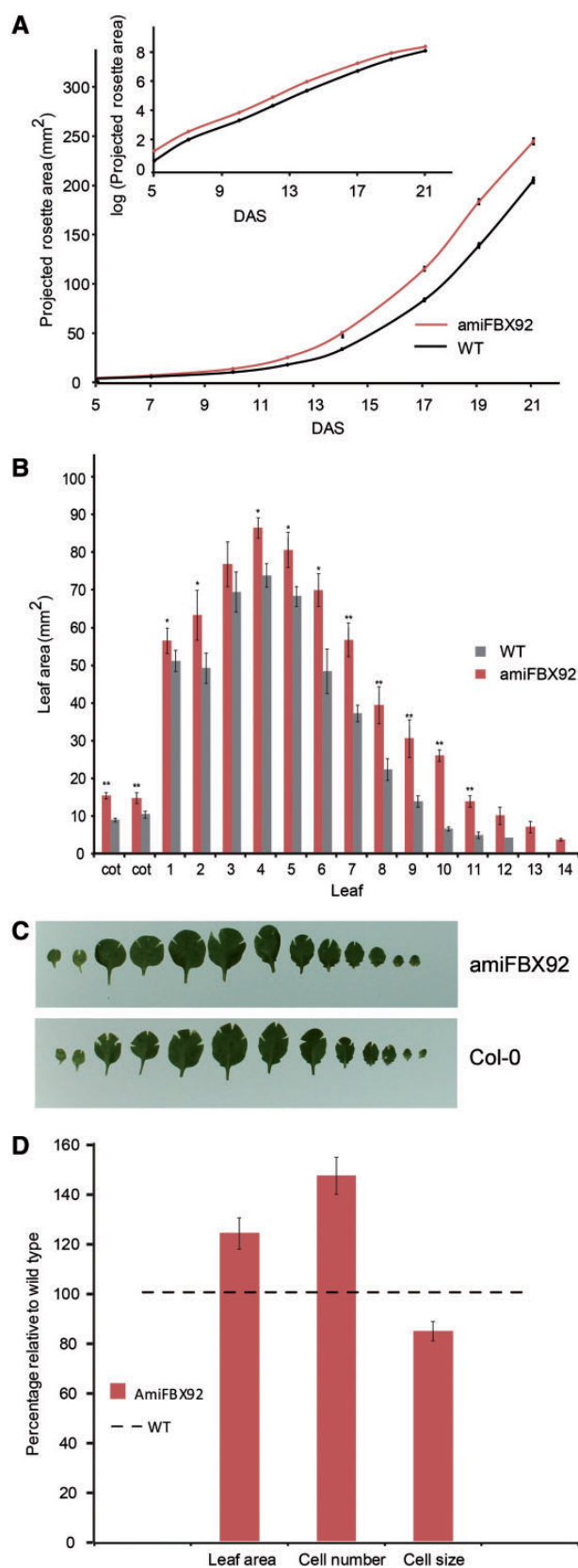


Fig. 3 Effect of *AtFBX92* down-regulation on rosette and leaf growth under standard conditions in vitro and cellular basis of the leaf size differences. (A) PRA of *amiFBX92* and the WT over time from 5 until 21 DAS. Plants were grown in vitro on standard medium. Inset: PRA in log scale. Values represent the mean \pm SE ($n_{\text{transgenic line}} = 22$, $n_{\text{WT}} =$

AtFBX92^{OE2} than for *AtFBX92*^{OE7} observed in vitro was completely lacking in soil; both lines showed an equal decrease of PRA (Supplementary Fig. S7A). Remarkably, growth of *AtFBX92*^{OE2} and *AtFBX92*^{OE7} in soil resulted in epinastic leaves (Fig. 6A), an effect that was not seen in vitro (Supplementary Fig. S1C).

The effect of *AtFBX92* on tolerance to mild drought stress

The reduction in PRAs of *AtFBX92*^{OE} and *amiFBX92* plants grown under osmotic stress conditions by adding mannitol to the medium was not significantly different from the reduction in PRA for WT plants grown under these conditions (Supplementary Fig. S3). Addition of mannitol to the growth medium is often used in lab settings as a proxy for osmotic stress. Because the response of soil-grown plants is closer to natural conditions, we also evaluated the effect of mild drought stress on soil-grown plants with altered *AtFBX92* levels and WT plants (Fig. 6B; Supplementary Fig. S8). We grew these plants on the WIWAM platform that allows, in addition to automated imaging, automated weighing and watering to control the applied water regime (see the Materials and Methods for details). In WT plants, the rosette size at 20 DAS was 34% reduced under mild drought conditions compared with well-watered conditions (Supplementary Fig. S7, S8). For the two *amiFBX92* lines, the reduction was comparable with the reduction in WT plants (35% and 33% reduction in PRA at 20 DAS for *amiFBX92-4* and *amiFBX92-13*, respectively). Overall, there was no significant difference in the PRA of *amiFBX92* and the WT in response to mild drought stress over time (Supplementary Fig. S8B), a result similar to that for the response to osmotic stress (Supplementary Fig. S3A). In contrast, the PRA of *AtFBX92*^{del} plants under mild drought stress was not significantly different from the PRA of WT plants (Supplementary Fig. S8C), whereas under standard conditions *AtFBX92*^{del} plants were significantly larger than the WT (Supplementary Fig. S7C). In contrast to the effect of down-regulating *AtFBX92* in standard conditions (Supplementary Fig. S7B), the PRA of *AtFBX92*^{OE} plants under mild drought stress was significantly larger (*AtFBX92*^{OE2}) or equal to (*AtFBX92*^{OE7}) the PRA of WT plants (Supplementary Fig. S8A). This is in contrast to what was found under osmotic stress (Supplementary Fig. S3A), for which there was no difference in response on PRA between WT and *AtFBX92*^{OE} plants.

Down-regulation of *AtFBX92* increases the cell division rate

To investigate the effect of the reduction in *FBX92* expression at the cellular level over time, leaf growth was analyzed kinematically (De Veylder et al. 2001). The first two initiated leaves of

Fig. 3 Continued

30). (B) Individual leaf size of 21-day-old WT and *amiFBX92* plants grown in vitro. Values represent the mean \pm SE ($n = 7$). Significant differences (Student's *t*-test): **P* < 0.05; ***P* < 0.01 relative to the WT. (C) Representative pictures from the measurements shown in (B). (D) Average area, pavement cell number and pavement cell size of leaves 1 and 2 at 21 DAS of *amiFBX92* plants relative to the WT. Values represent the mean \pm SE ($n = 3$).

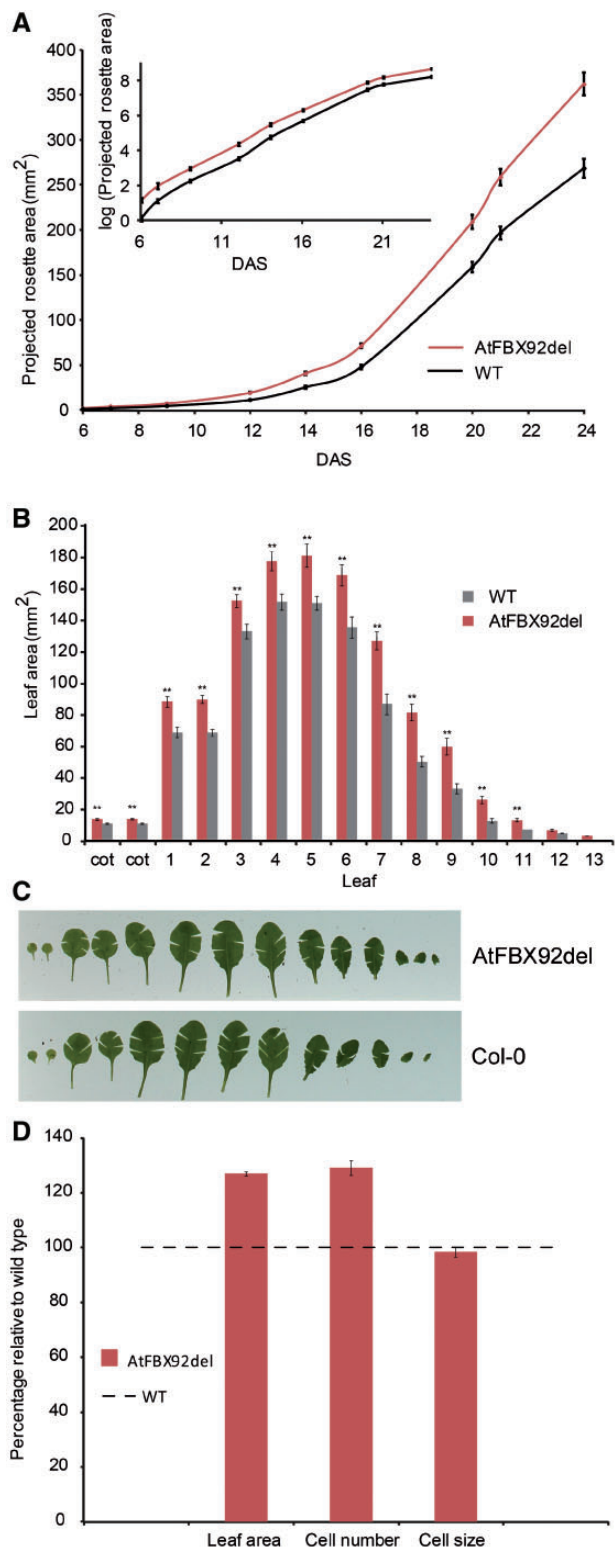


Fig. 4 Effect of *AtFBX92^{del}* expression on rosette and leaf growth under standard conditions in vitro and cellular basis of the leaf size differences. (A) PRA of *AtFBX92^{del}* and the WT over time from 6 until 24 DAS. Plants were grown in vitro on standard medium. Inset: PRA in log scale. Values represent the mean \pm SE ($n_{\text{transgenic line}} = 46$, $n_{\text{WT}} = 58$). (B) Individual leaf size of 20-day-old WT and *AtFBX92^{del}* plants grown in soil. Values represent the mean \pm SE ($n = 12$). Significant differences (Student's *t*-test): $**P < 0.01$ relative to the WT. (C) Representative pictures from

amiFBX92 and WT plants grown in vitro were harvested daily from 5 until 21 DAS for quantitative image analysis of leaf blade area, and cell number and cell size of the abaxial epidermis (Fig. 7). The leaf size of *amiFBX92* plants was not significantly different from that of the WT until 7 DAS, when the difference became significant (Fig. 7A, inset). At maturity, *amiFBX92* leaves were approximately 30% larger than those of the WT. The cell area remained constant until 8 DAS, i.e. during the period of cell division, and then increased exponentially (Fig. 7C). Transgenic and WT plants followed the same trend of cell area increase over time, suggesting that the down-regulation of *AtFBX92* did not affect cell expansion. The cell number per leaf, however, was strongly increased in *amiFBX92* plants compared with the WT (Fig. 7B); at maturity the difference was approximately 25%, supporting our previous data. Average cell division rates of the whole leaf were estimated from the exponential increase in cell number. Cell cycle duration was higher in *amiFBX92* plants (16 ± 2 h) than in the WT (20 ± 1 h) from 5 until 7 DAS, after which they followed the same trend, reaching zero at day 14 in the first leaf pair (Fig. 7D). Thus, the increased final leaf size in *amiFBX92* is most probably due to augmented cell division rates during very early stages of leaf development.

Quantitative reverse transcription-PCR (qRT-PCR) of cell cycle genes during early phases of development in *amiFBX92*

Because the cell proliferation rate is affected in *amiFBX92* plants, we examined the expression of several cell cycle genes. Total RNA was isolated from the first leaf pair of WT and *amiFBX92* plants at 7 and 8 DAS, the first time points at which cell numbers were significantly different, and subjected to qRT-PCR. The two major cell cycle phase transitions, the G_1 to S and G_2 to M, are controlled by the consecutive action of cyclin-CDK complexes (Inzé and De Veylder 2006). We found that transcript levels of genes involved in the G_1 to S phase transition, such as the D-type cyclins, and genes involved in the G_2 to M transition, such as *CYCB1;1*, *CDKB1;1* and *CDKB1;2*, were increased in *amiFBX92* lines (Fig. 8). Surprisingly, in addition to these positive cell cycle regulators, the expression levels of some of the negative cell cycle regulators—ICK/KIP/CIP-RELATED PROTEINS (ICK/KRPs), SIAMESE (SIM) and SIM-related (SMR) proteins—were also up-regulated in *amiFBX92* leaves (Fig. 8). We could confirm this in an additional *amiFBX92* line, *amiFBX92-13* (Supplementary Fig. S9A). Moreover, expression levels of both positive and negative cell cycle genes were down-regulated and up-regulated in proliferating *AtFBX92^{OE}* and *AtFBX92^{del}* leaves, respectively, compared with WT leaves, supporting the increase in expression of cell cycle genes in proliferative leaves with reduced *AtFBX92* activity and/or expression levels (Supplementary Fig. S9B, C).

the measurements shown in (B). (D) Average area, pavement cell number and pavement cell size of leaves 1 and 2 at 20 DAS of *AtFBX92^{del}* plants relative to the WT. Values represent the mean \pm SE ($n = 3$).

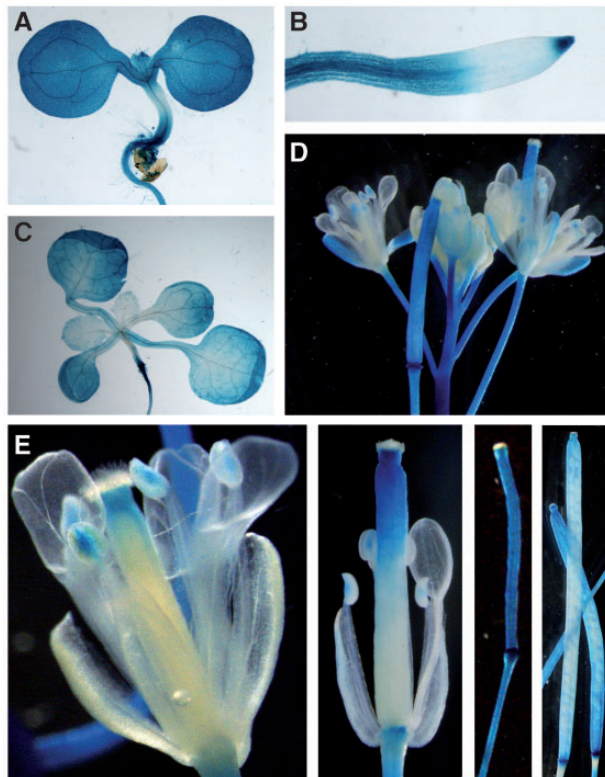


Fig. 5 Expression of the pAtFBX92:GFP:GUS reporter gene at different developmental stages. (A) Seedling shoot at 6 DAS. (B) Main root. (C) Seedling shoot at 13 DAS. (D) Flower cluster of a 6-week-old plant. (E) Carpels and developing siliques of a 6-week-old plant.

Discussion

In this study, we present the characterization of a new plant-specific F-box-containing protein we designated FBX92. Reducing *AtFBX92* expression levels using an amiRNA approach resulted in plants that developed larger cotyledons and leaves, whereas an opposite effect was seen in plants overexpressing *AtFBX92*. We showed that this difference in leaf size was primarily due to an effect on cell number, compensated slightly by an effect on cell size. The cell number in a plant organ is determined by three things: first, the cell number in the leaf primordia recruited from the shoot apical meristem (SAM); secondly, the cell proliferation rate; and thirdly, the cell proliferation duration (Gonzalez et al. 2012). Because the leaf primordia of *amiFBX92* plants were equal in size to those of WT plants at the first time point of analysis (5 DAS), we can assume that the number of cells recruited from the SAM for leaf initiation is not altered. Detailed kinematic analysis of the first *amiFBX92* leaf pair indicated that the observed increase in leaf size was specifically due to an enhanced rate of cell division during the first days of leaf development, which was maintained until maturity. Moreover, the average duration of the cell cycle decreased from 20 h in the WT to about 16 h in *amiFBX92*. There are only a few genes known to increase the rate of cell division in developing leaves. Overexpression of the APC/C subunit APC10 accelerates the average cell cycle duration from 21 to 19 h (Eloy et al. 2011).



Fig. 6 Effect of *AtFBX92* misexpression on leaf size of plants grown in soil under standard and drought stress conditions. Representative pictures of 20-day-old *amiFBX92*, *AtFBX92^{del}*, *AtFBX92^{OE}* and Col-0 plants grown in soil under standard conditions (A) and under mild drought stress (B).

It is likely that CDC27a, another regulator of APC/C, also affects the rate of cell division (Rojas et al. 2009). The observation that cell division is enhanced in the *amiFBX92* plants was further supported by the enhanced expression level of several cell cycle genes in *amiFBX92* and their decreased expression levels in *AtFBX92^{OE}* plants during this early leaf development. In agreement with our observations, CDKA levels in the roots of different *Arabidopsis* ecotypes are positively correlated with cell division rates (Beemster et al. 2002). Also in cell suspension cultures, the expression levels of A-, B- and D-type cyclins and *CDKB1;1* coincided with cell division rates (Richard et al. 2001). For several genes shown to function in organ size

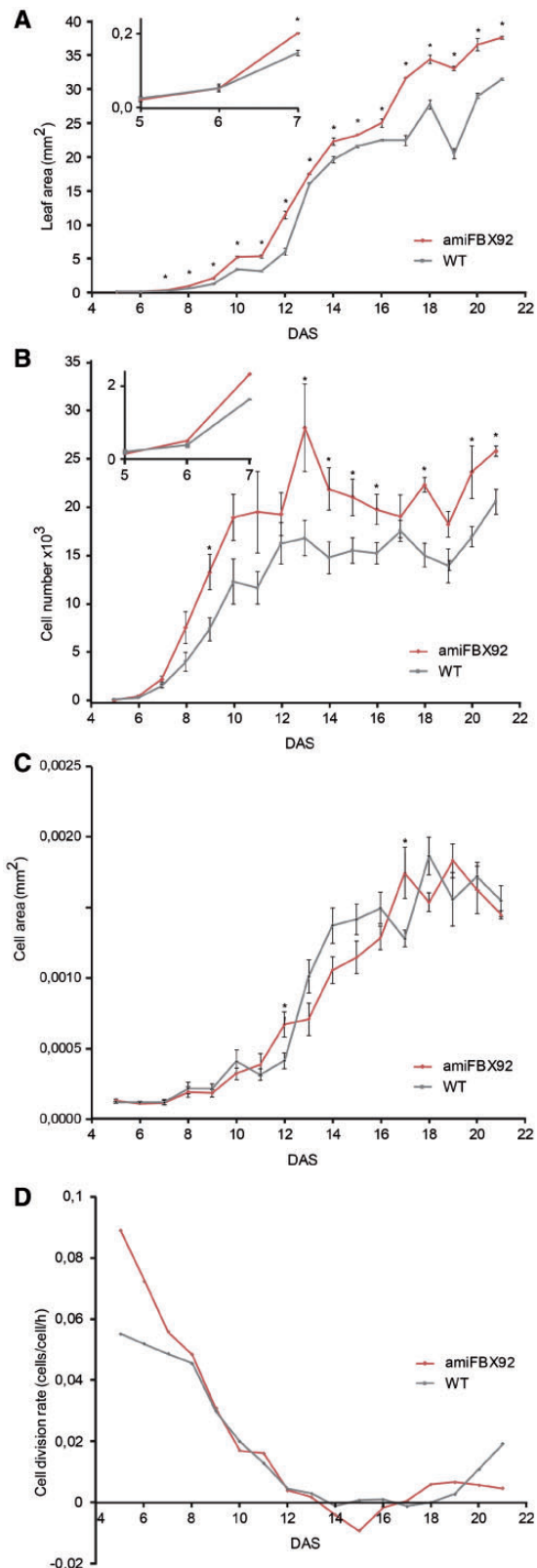


Fig. 7 Kinematic analysis of the first leaf pair of *amiFBX92* and WT plants grown in vitro from 4 to 22 DAS. (A) Leaf area. Inset: measurements at 5–7 DAS. (B) Cell number. Inset: measurements at 5–7 DAS. (C) Cell area. (D) Cell division rate. Values represent the mean \pm SE ($n = 4-6$) for (A), (B) and (C); significant differences (Student's *t*-test): * $P < 0.05$; ** $P < 0.01$ relative to the WT.

determination by altering cell division rates, it has been shown that, when altering their expression, the expression of cell cycle-regulating genes was also affected (Achard and Genschik 2009, Lee et al. 2009, Rojas et al. 2009, Eloy et al. 2011). We found both positive and negative cell cycle regulators up-regulated in *amiFBX92* proliferative leaves, although it has been shown that constitutive expression of the negative regulators *KRP* and *SMR* results in growth retardation (Verkest et al. 2005, Churchman et al. 2006, Hudik et al. 2014). This discrepancy might be due to the specific time points of our analysis at which the leaf was fully proliferative. It is very possible that in later stages of leaf development, the levels of these positive and negative regulators are not affected or show different trends in *amiFBX92* or *AtFBX92^{OE}*. Also, in cell suspension cultures, the expression levels of negative cell cycle regulators peak with the highest cell division rate (Richard et al. 2001), and in animal systems some of the KRPs have a role in assembling CDK–cyclin complexes (Sherr and Roberts 1999). In agreement with the kinematic analysis, the up-regulation of both positive and negative regulators in proliferative leaves might suggest that there is no effect on the timing of the transition from cell division to cell expansion. Moreover, because genes involved in the G₁ to S phase transition as well as genes involved in the G₂ to M transition show differential expression, we can speculate that reducing *AtFBX92* activity results in a faster progression through both S-phase and mitosis. In agreement with this, *AtFBX92* expression levels seem not to be specific for one of the phases of the cell cycle in cell cultures (Menges et al. 2003). Possibly, the effect of altered *AtFBX92* levels on the expression of cell cycle genes is indirect; it might be that *AtFBX92* targets a currently unknown growth-regulating factor for proteolysis. For instance, the SCF complex containing SLEEPY1 (SLY1) is an F-box protein that affects cell division indirectly by gibberellin-mediated degradation of the growth-repressing DELLA proteins (Achard et al. 2009).

Although the cell cycle and the proteolytic processes are conserved between yeast, animals and plants, only a few plant F-box proteins have been identified to modulate the G₁ to S phase transition of the cell cycle (reviewed by Genschik et al. 2014). *SKP2A* and *SKP2B* act as positive and negative regulators, respectively, of root cell division by targeting specific cell cycle regulators for degradation (del Pozo et al. 2006, Ren et al. 2008). Plants with reduced *SKP2A* and *SKP2B* expression levels exhibit only mild phenotypes (del Pozo et al. 2006, Manzano et al. 2012). Also the effect of *AtFBX92* perturbation on plant development is rather mild. This might be due to only limited variation of expression levels in the *amiFBX92* and *AtFBX92^{OE}* lines compared with WT plants. Additionally, it is possible that this F-box protein is also regulated at the post-transcriptional level. For instance, it has been shown that many F-box proteins are intrinsically unstable because they are often themselves targeted for degradation by the ubiquitin–proteasome pathway (Jurado et al. 2008, Marrocco et al. 2010). This would be in agreement with the fact that we could not stably express a tagged version of this F-box protein, either in cell cultures or in seedlings (data not shown). Also, it is likely that there are redundant mechanisms governing the different steps of cell

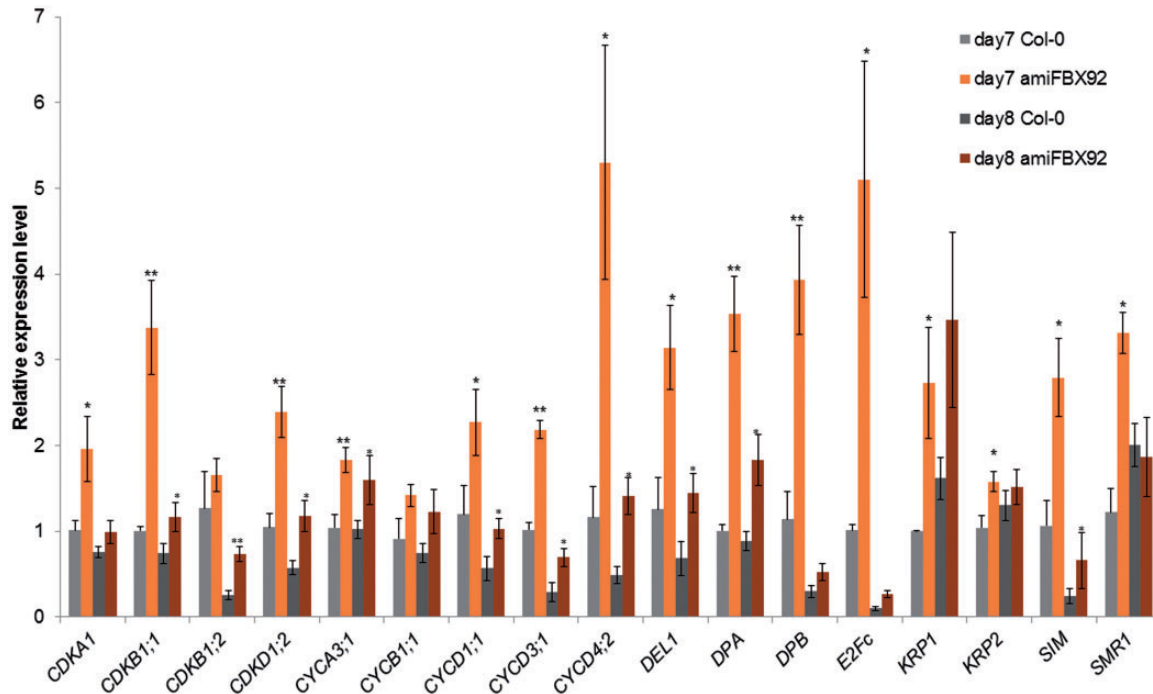


Fig. 8 Relative expression levels of cell cycle genes in *amiFBX92* in the first leaf pair compared with the WT at 7 and 8 DAS as determined by qRT-PCR. Values were normalized against the expression level of the housekeeping gene and represent the main expression levels of the indicated transcripts of three biological repeats \pm SE. Significant differences (Student's *t*-test): **P* < 0.10; ***P* < 0.01 relative to the WT.

division; potentially there might be a partial compensation by other proteins and protein complexes, which has also been suggested for other F-box proteins (Dharmasiri et al. 2005, Qiao et al. 2009, An et al. 2010, Schumann et al. 2011, Manzano et al. 2012). Recently, an F-box protein, FBL17, was identified as an important regulator of the cell cycle at different stages of plant development (Gusti et al. 2009, Zhao et al. 2012, Noir et al. 2015). Loss of *FBL17* function drastically impaired plant development during the sporophytic life cycle as well as during gametogenesis by reducing cell proliferation, due to an increased stability of the cell cycle inhibitor KRP2, although the drastic phenotype suggests that this F-box protein also targets other substrates for degradation (Noir et al. 2015).

The expression of *AtFBX92* was examined in a large number of published microarray data sets using the Arabidopsis eFP Browser (www.bar.utoronto.ca) and Genevestigator (<https://www.genevestigator.com>), showing that expression was rather weak in most tissues except in pollen. Analysis of *GUS* reporter lines confirmed that *AtFBX92* is expressed in almost all sporophytic tissues, in tissues with low proliferation rates, but not in root meristems. *GUS* expression was also visible in reproductive tissues, i.e. in sepals, style, carpels, developing siliques and pollen grains, although gametogenesis and seed set were not affected in *amiFBX92*, *AtFBX92^{OE}* and *AtFBX92^{del}* plants.

Ectopic expression of *ZmFBX92* in Arabidopsis and of the deletion mutant *AtFBX92^{del}*, both lacking the F-box-associated interaction domain, resulted in plants with larger leaves, comparable with the phenotype of *amiFBX92*. We hypothesize that these ectopic or mutant proteins bind to the SCF complex and inhibit binding of the native *AtFBX92* protein, in that way preventing the ubiquitination of the target proteins, because the F-box-associated

interaction domain that most probably recruits the target is lacking in these ectopic or mutant proteins. Overexpression of *ZmFBX92* in maize, however, has no apparent phenotype. Because the native *ZmFBX92* lacks the F-box-associated interaction domain, it is possible that this protein must interact with a protein containing an F-box-associated interaction domain to exert its function. In maize, there are 17 genes with an F-box-associated interaction domain, whereas there are 283 in Arabidopsis (bioinformatics.psb.ugent.be/plaza/). However, only two of the 17 genes in maize, *GRMZM2G055789* and *GRMZM2G083000*, lack the F-box domain and thus are potential candidates for interaction with *ZmFBX92* and possibly other F-box domain-containing proteins. This might explain why the number of F-box proteins in Arabidopsis is much larger, about double, than the number of F-box proteins identified in maize (Risseuw et al. 2003, Jia et al. 2013). Moreover, more than half of the maize F-box proteins contain only the F-box domain without other known motifs (Jia et al. 2013), whereas in Arabidopsis only 14% of the F-box proteins have no additional domains (Risseuw et al. 2003). Perhaps the lower number of F-box proteins in maize is partially compensated by the possibility of different combinations of F-box proteins and F-box-interacting domain proteins to target other proteins for degradation or play a role under other conditions.

The effects of altering *AtFBX92* levels on leaf size were largely comparable in vitro and in soil under well-watered conditions. However, opposite effects on growth upon *AtFBX92* overexpression were obtained under osmotic stress and drought stress. Under mild drought stress applied in soil, leaves of *AtFBX92^{OE}* plants were larger than those of WT plants, whereas under standard or mild in vitro osmotic stress conditions, they grew more slowly than the WT. Although osmotica such as mannitol are

often used to mimic drought stress responses, their relevance is debatable (Verslues et al. 2006, Lawlor 2013). Our data suggest that *AtFBX92* might be involved in the mild drought stress response, but not in the osmotic stress response. It has often been seen that enhancing drought tolerance by altering gene expression levels, as shown here for *AtFBX92^{OE}* plants, results in growth inhibition and a significant yield penalty (Yang et al. 2010). Leaves of *amiFBX92* plants were significantly larger than those of the WT under standard conditions in vitro and in soil, and in stress conditions when grown on mannitol or under mild drought conditions. There was no significant difference in effect of both stresses compared with standard conditions, implying that *AtFBX92* plays no role in the osmotic or drought stress response, in contrast to what is suggested by the results for *AtFBX92^{OE}* plants. Expression levels of *AtFBX92* do not vary significantly under mild drought stress and osmotic stress (Skirycz et al. 2011a, Clauw et al. 2015, Dubois et al. 2017), supporting that the observed enhanced drought tolerance in *AtFBX92^{OE}* plants is rather an indirect effect of changed *AtFBX92* levels. Alternatively, functionally redundant proteins may exist that shield the effect that reduced *AtFBX92* levels have on drought tolerance.

Strikingly, leaves of *AtFBX92^{OE}* plants grown in soil, under both standard and mild drought stress conditions, were folded downwards, whereas the shape of *AtFBX92^{OE}* and WT leaves grown in vitro was indistinguishable. Possibly, epinasty of the leaf lamina in *AtFBX92^{OE}* plants only appears in specific conditions, for instance under lower relative humidity or specific light conditions (Takemiya et al. 2005, de Carbonnel et al. 2010). Epinastic leaves result from a difference in cell division rate between abaxial and adaxial cells (Romano et al. 1995). In *amiFBX92* plants, although no effect on leaf flattening was observed, we could show that the increased leaf size was due to an increased cell division rate. Also other genes affecting leaf development and final leaf size have been identified that influence leaf curling, e.g. *BREVIS RADIX* (Beuchat et al. 2010), several members of the *TEOSINTE BRANCHED1/CYCLOIDEA/PCF* (*TCP*) transcription factor family (Schommer et al. 2008), *jaw-D* (Palatnik et al. 2003), *PEAPOD* (Gonzalez et al. 2015) and *TCP* Interactor containing EAR motif protein1 (Tao et al. 2013), often linked to hormone biosynthesis or signaling. Potentially, *AtFBX92* does not affect cell cycle genes directly, but by regulating hormone signaling.

In conclusion, we identified a new F-box gene that is important for vegetative growth. This gene acts as a negative regulator of growth, as indicated by the effect on leaf size when altering *AtFBX92* levels, by affecting cell division rates and expression levels of cell cycle genes. Future experiments are needed to identify putative substrates that might have a role in the regulation of organ growth.

Materials and Methods

Cloning and generation of transgenic plants

The coding region of maize F-box protein GRMZM2G059799_T02 (*ZmFBX92*) was amplified with Phusion High-Fidelity DNA polymerase (Thermo Fischer Scientific) from cDNA generated from leaf tissue of the maize inbred line B73.

The PCR fragment was introduced by recombination via the *attBattP* recombination sites into pDONRTM221 using the Gateway system (Invitrogen Life Technologies). Next, the *ZmFBX92* coding region and pBdEF1 α (Coussens et al. 2012) were transferred to the binary vector pBbm42GW7 (Anami et al. 2010) (<https://gateway.psb.ugent.be>) using a multisite Gateway approach (pBdEF1 α :*ZmFBX92*). Additionally, the *ZmFBX92* coding region was also introduced in the binary vector pK7GW2 (<https://gateway.psb.ugent.be>), under control of the CaMV 35S promoter (pCaMV35S:*ZmFBX92*) for the generation of Arabidopsis transgenic plants (Karimi et al. 2007).

The closest orthologous gene of *ZmFBX92* in Arabidopsis was identified using PLAZA (Proost et al. 2015) (<https://plaza.psb.ugent.be>) and the coding region of this gene, At3g07870 (*AtFBX92*), was amplified with Phusion High-Fidelity DNA polymerase (Thermo Fischer Scientific) from cDNA generated from leaf tissue of Arabidopsis ecotype Col-0. In addition, a fragment consisting of the first 492 bp starting from the ATG start codon, containing the F-box domain, was amplified by PCR for further cloning. The Gateway system was used to introduce the obtained PCR fragments via recombination into pDONR221 (Invitrogen Life Technologies), followed by recombination via the *attLatR* sites into binary vector pK7GW2 (<https://gateway.psb.ugent.be>) into which a cassette containing the seed-specific napin promoter (Ellerström et al. 1996) driving GFP was introduced, further indicated as pK7GW2^{napin}, to allow the selection of transgenic seeds based on GFP expression in the seed. The generated constructs, pCaMV35S:*AtFBX92* and pCaMV35S:*AtFBX92^{del}*, were subsequently transformed into Arabidopsis.

For silencing of *AtFBX92*, a pCaMV35S:*AtFBX92-amiRNA* construct was designed using the pRS300 plasmid as described before (Ossowski et al. 2008), inserted in pDONRTM221 (Invitrogen Life Technologies) and sequenced. Next, the DNA construct was transferred to pK7GW2^{napin} by recombination.

For analysis of the *AtFBX92* promoter, a 1,362 bp fragment upstream of the ATG start codon was amplified with Phusion High-Fidelity DNA polymerase (Thermo Fischer Scientific) from Arabidopsis Col-0 genomic DNA, cloned into pDONRTM221 (Invitrogen Life Technologies) and transferred to the pFAST-G04 binary vector (Shimada et al. 2010) (<https://gateway.psb.ugent.be>) to generate the p*AtFBX92*:GFP:*GUS* construct. Primers used for cloning are summarized in Supplementary Table S1.

pBdEF1 α :*ZmFBX92* was introduced into maize cultivar B104 by *Agrobacterium tumefaciens* transformation of immature embryos as described before (Coussens et al. 2012).

pCaMV35S:*ZmFBX92*, p35S:*AtFBX92*, p35S:*AtFBX92^{del}*, p35S:*AtFBX92-amiRNA* and p*AtFBX92*:GFP:*GUS* constructs were transformed into *A. tumefaciens* strain C58C1 Rif^R harboring the plasmid pMP90, followed by transformation into Arabidopsis Col-0 using the floral dip protocol (Clough and Bent 1998).

Maize growth analysis

Maize plants were grown in controlled growth chamber conditions (24 °C, 55% relative humidity, light intensity of 170 mmol m⁻² s⁻¹ photosynthetic active radiation, in a 16 h/8 h day/night cycle). Pot weight was determined daily and water was added to 100% of the initial water content under well-watered conditions, and to 70% of the initial water content under mild drought stress conditions. Leaf size-related phenotypic observations were performed as described before (Baute et al. 2015).

Arabidopsis growth analysis

Arabidopsis plants were grown in vitro on round Petri dishes containing half-strength Murashige and Skoog medium supplemented with 1% (w/v) sucrose at 21 °C, light intensity of 70 mmol m⁻² s⁻¹ photosynthetic active radiation, in a 16 h/8 h day/night cycle. To subject plants to mild osmotic stress, seeds were germinated on medium containing 25 mM d-mannitol (Sigma-Aldrich) (Claeys et al. 2014).

Rosette growth over time was determined for plants grown in vitro at a density of one plant per 4 cm² by photographing the plates three times a week from 5 to 6 DAS until 21–24 DAS (dependent on the experiment) and calculating the PRA with IMAGEJ software version 1.46 (<http://rsb.info.nih.gov/ij/>). Relative growth rates were calculated as the log of the PRA over time.

Arabidopsis growth analysis in soil was performed on the automated phenotyping platform WIWAM (Skirycz et al. 2011b) in a growth chamber under controlled conditions (21 °C, 55% relative humidity, light intensity of 100–120 mmol m⁻² s⁻¹ photosynthetic active radiation, in a 16 h/8 h day/night cycle). The water content of the soil was kept constant at 2.19 g water g⁻¹ dry soil for control plants during the entire experiment. For mild drought-treated plants, from 10 DAS on, water was withheld until a soil water content of 1.19 g⁻¹ water g⁻¹ dry soil was reached, and kept at this level until 21 DAS. Images of the rosettes were taken daily from 6 DAS until 20 DAS, and PRA and relative growth rates were determined as explained before.

For rosette leaf area measurements, seven seedlings grown in vitro or in soil for 21 or 22 d (dependent on the experiment) were dissected and spread on agar plates according to their position in the rosette. Agar plates were photographed and individual leaf area was determined using IMAGEJ software.

Kinematic analysis

Kinematic analysis was performed as described before (De Veylder et al. 2001) on the first true leaf pair of 12 *amiFBX92* and Col-0 plants grown in vitro from 5 DAS until 21 DAS. Briefly, leaves 1 and 2 were harvested daily, cleared in 100% ethanol, mounted in lactic acid on microscope slides and photographed. Leaf area of each leaf was measured using IMAGEJ. To determine the average cell area at each time point, 50–100 abaxial epidermal cells of 3–5 leaves were drawn with a Leica microscope fitted with a drawing tube and a differential interference contrast objective, and the average cell area was determined with IMAGEJ. From the leaf area and average cell area, cell numbers per leaf were calculated. Average cell division rates for the whole leaf were determined as the slope of the log 2-transformed cell number.

Using the same protocol, we determined the size, average cell number and average cell size of mature leaves 1 and 2 (*amiFBX92*, *AtFBX92^{del}* and the respective Col-0) or leaf 3 (*ZmFBX92^{OE}*, *AtFBX92^{OE}* and respective Col-0).

RNA extraction and qRT-PCR

Tissue for RNA extraction was flash-frozen in liquid nitrogen immediately upon harvest to avoid degradation. To analyze expression levels of the cell cycle genes in fully proliferative tissue, about 50 *amiFBX92* and WT plants were sampled and flash-frozen at 7 and 8 DAS, after which cooled RNAlater-ICE (Ambion) was added to the samples. Samples were kept at –20 °C for 1 week to allow the RNAlater-ICE to penetrate the tissue. Leaves 1 and 2 were dissected under a binocular microscope while the samples were kept on dry ice before grinding.

Total RNA was extracted from frozen material with TRIzol (Invitrogen) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Healthcare) to eliminate residual genomic DNA possibly present after RNA extraction. First-strand cDNA was synthesized starting from 1 µg of total RNA using iScriptTM (Biorad) according to the manufacturer's instructions. cDNA was amplified on a LightCycler480 (Roche Diagnostics) in 384-well plates with LightCycler 480 SYBR Green I Master (Roche) as described by the manufacturer. Gene-specific primers were designed with the Beacon DesignerTM software and are summarized in Supplementary Table S1. The specificity of the amplification was determined by analyzing the melting curves. Normalization was done against the maize 18S rRNA or Arabidopsis *ACTIN1* gene, and PCR efficiency was taken into account using geNorm (Vandesompele et al. 2002). Relative expression levels were calculated based on the $\Delta\Delta$ cycle threshold method (Livak and Schmittgen 2001). Data presented for testing expression levels of *AtFBX92* in *AtFBX92^{OE}*, *amiFBX92*, *AtFBX92^{del}* and *ZmFBX92* in Arabidopsis and maize, respectively, were from triplicates. Data presented for cell cycle gene expression levels were from duplicates of three biological repeats. Values for the three biological repeats were used for statistical analysis.

Histochemical staining of GUS activity

pAtFBX92:GFP:GUS and *pCYCB1;1:DB-GUS* Arabidopsis plants grown in vitro were harvested daily from 5 to 13 DAS, followed by incubation in heptane for 5 min. After removal of the heptane, plants were incubated in 5-bromo-4-

chloro-3-indolyl- β -glucuronide (X-Gluc) buffer [100 mM sodium phosphate pH 7, 10 mM EDTA, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.5 g l⁻¹ X-gluc, 1% dimethylsulfoxide (DMSO)] and incubated for 6 h at 37 °C after vacuum infiltration for 10 min. Plants were cleared in 100% (v/v) ethanol until Chl was removed, and kept in 90% lactic acid. Samples were photographed under a differential interference contrast microscope (Leica).

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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