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Original Paper

Overexpression of *LOV KELCH PROTEIN 2* enhances cell elongation and increases cell number and ploidy in the hypocotyl of *Arabidopsis thaliana*

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Abstract LOV KELCH PROTEIN 2 (LKP2) belongs to a group of blue light receptors in *Arabidopsis thaliana* that possess 1 light, oxygen, or voltage (LOV) domain, 1 F-box motif, and 1 kelch repeat domain. Members of this group regulate both the circadian clock and photoperiodic flowering. Transgenic *Arabidopsis* plants overexpressing a fusion of the genes for green fluorescent protein (GFP) and LKP2 exhibited long hypocotyls and arrhythmicity under constant light and late flowering under long-day conditions, as previously reported for *LKP2*-overexpressing *Arabidopsis* plants. The GFP-*LKP2*-overexpressing plants with elongated hypocotyls had more cortical cells than control plants but the same number of epidermal cells. Further, the cells in the hypocotyls of GFP-*LKP2*-overexpressing plants, especially in the middle regions of the epidermis and cortex, were longer and possessed larger nuclei, with increased DNA content. Therefore, *LKP2* overexpression induces cell elongation and increases in cell number and ploidy in the *Arabidopsis* hypocotyls.

Key words: *Arabidopsis*, cell elongation, hypocotyl, LKP2, ploidy.

Plants use light as a signal that lets them adapt to their environment and as an energy source for photosynthesis. Light regulates germination, de-etiolation, leaf and stem growth, flowering, entrainment of circadian rhythms, stomatal opening, chloroplast movement, and anthocyanin synthesis, among other processes (Jiao et al. 2007; Mancinelli 1990; Millar 2004).

In *Arabidopsis*, hypocotyl growth is affected by both the quality and the quantity of light. After germination, the absence of light induces hypocotyl elongation, whereas the presence of blue, red, or far-red light inhibits it (Gendreau et al. 1998). Numerous genes and proteins, including photoreceptors, are involved in this induction and inhibition of hypocotyl growth (Parks et al. 2001). Light-activated phytochromes enter the nucleus and regulate the transcription of light-responsive genes via their interaction with several transcription factors (TFs), such as phytochrome-interacting factors (PIFs) and PIF3-like proteins (PILs) (Bae and Choi 2008; Castillon et al. 2007). For example, light-activated phytochromes bind to PIF3, an inhibitory TF for photomorphogenesis, in the nucleus, followed by the degradation of PIF3 and subsequent activation of certain light-responsive genes (Duek and Fankhauser 2005; Nozue and Maloof 2006). The direct interaction of phytochromes with the TFs in the nucleus is an important step in phytochrome regulation of hypocotyl elongation (Bauer et al. 2004; Nozue and Maloof 2006). Cryptochromes can interact with CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1: an E3 ubiquitin ligase that controls proteasome-dependent degradation of ubiquitinated proteins) and regulate the activity of protein degradation (Seo et al. 2004; Yang et al. 2001). For example, photoactivated cryptochrome 1 represses COP1-mediated degradation of LONG HYPOCOTYL 5 (HY5), a basic region/leucine zipper motif (bZIP) TF that regulates the transcription of several genes that function in photomorphogenesis (Yi and Deng 2005).

The FKF/LKP/ZTL family is a group of photoreceptors in *Arabidopsis*. The proteins of this family—namely, FLAVIN-BINDING KELCH REPEAT F-BOX 1 (FKF1), LOV KELCH PROTEIN 1 (LKP1)/ZEITLUPE

Abbreviations: CAB2, CHLOROPHYLL A/B-BINDING PROTEIN 2; CCR2, COLD-CIRCADIAN RHYTHM-RNA BINDING 2; Col, Columbia; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; GFP, green fluorescent protein; GFP-LKP2ox, *p35S:GFP-LKP2*-overexpressing; GFPox, *p35S:GFP*-overexpressing; LUC, luciferase.

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(ZTL), and LOV KELCH PROTEIN 2 (LKP2)–possess 1 light, oxygen, or voltage (LOV) domain, 1 F-box motif, and 1 kelch repeat domain (Kiyosue and Wada 2000; Nelson et al. 2000; Schultz et al. 2001; Somers et al. 2000). The LOV domain binds to flavin mononucleotide and displays photochemical properties analogous to those of phototropin LOV domains (Demarsy and Fankhauser 2009). The F-box motif is involved in the formation of the Cul1–Rbx1–Skp1–F-box (SCF) complex, which functions as an E3 ubiquitin ligase (Vierstra 2003). The kelch repeat forms a β-propeller structure used in protein–protein interactions (Adams et al. 2000). The FKF/LKP/ZTL family proteins determine the period of circadian oscillation, regulate photoperiodic flowering, and are involved in light-controlled hypocotyl elongation (Baudry et al. 2010; Fornara et al. 2009). *fkf1* mutants produce a short hypocotyl under continuous blue or red light, and both *LKP2*-overexpressing plants and *ZTL*-overexpressing plants produce elongated hypocotyls under continuous blue, red, or white light (Kiyosue and Wada 2000; Nelson et al. 2000; Schultz et al. 2001). These results suggest that proteins of this family do not function as blue light receptors for the light-induced inhibition of hypocotyl growth.

The circadian clock is another factor that affects hypocotyl growth in *Arabidopsis*. It controls hypocotyl growth immediately upon germination (Dowson-Day and Millar 1999). Under constant dim light, the hypocotyl elongation rate is fastest at subjective dusk, and stops close to subjective dawn (Dowson-Day and Millar 1999). Studies using clock mutants revealed the importance of the circadian clock in hypocotyl growth. There is a strong correlation between circadian and hypocotyl mutant phenotypes. Arrhythmic mutants tend to abolish the daily arrest in hypocotyl growth in continuous light, while shorter- or longer-period mutants tend to alter either the duration or the amplitude of the growth phase (Nozue and Maloof 2006). Since FKF/LKP/ZTL family proteins are involved in circadian clock regulation, they contribute to hypocotyl growth regulation apparently via the circadian clock function. However, to understand the precise mechanisms underlying the regulation of hypocotyl growth by these proteins, more studies are needed.

In this study, we characterized the hypocotyl elongation phenotype in *LKP2*-overexpressing plants to improve our understanding of the mechanism that regulates hypocotyl growth caused by *LKP2* overexpression. We showed that cell elongation was enhanced and cell numbers and ploidy were increased in *LKP2*-overexpressing hypocotyls.

Materials and methods

Plant material and growth conditions

We used *Arabidopsis thaliana* Columbia (Col) in this study. We included *p35S:GFP-LKP2*-overexpressing (GFP-LKP2ox) and *p35S:GFP*-overexpressing (GFPox) plants (Yasuhara et al. 2004) to ensure that the GFP did not influence our results. To measure hypocotyl characteristics, we sowed seeds on germination medium (GM) agar (0.8% w/v) (Valvekens et al. 1988) and incubated them at 4°C for 3 days in the dark and then at 22°C for 5 days under continuous white light (90–100 μmol m^{-2} s^{-1}). We used Scion Image software (http://www.scioncorp.com/pages/scion_image_windows.htm) to measure hypocotyl length, cell length, and cell number in images taken by microscope (BX51; Olympus, Tokyo, Japan). We measured protruding epidermal cells and cortical outer-layer cells. The cells of the top, middle, and bottom parts were defined as follows. The top part was 3 epidermal and 6 cortical cells from the cotyledon–hypocotyl junction. The middle part was 3 epidermal and 6 cortical cells midway between the cotyledon–hypocotyl junction and the hypocotyl–root junction. The bottom part was 3 epidermal and 6 cortical cells from the hypocotyl–root junction (Supplemental Figure 1). To determine the flowering time, we grew the plants in soil under long-day (16-h light/8-h dark) conditions at 22°C under white light (90–100 μmol m^{-2} s^{-1}), and compared the number of days to flowering among the plants.

Bioluminescence analysis

To introduce the *pCAB2:LUC* (Millar et al. 1992) or *pCCR2:LUC* (Strayer et al. 2000) construct, we crossed lines homozygous for these constructs with lines homozygous for *GFP-LKP2* or *GFP* overexpression. The circadian bioluminescence rhythm was measured as described previously (Yamamoto et al. 2003). F_1 seedlings were grown on GM agar plates at 22°C under 12-h light (20 μmol m^{-2} s^{-1})/12-h dark for 4 days and then transferred to continuous light. The luciferase (LUC) activity of the individual seedlings was monitored with an automated scintillation counter (TopCount; Packard, Tokyo, Japan).

DAPI staining

Five-day-old seedlings were stained with 1% 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution (Wako Pure Chemical Industries, Osaka, Japan) for 30 min. Next, the cells from the upper, middle, and lower parts of the hypocotyls were visualized under a microscope (BX51; Olympus, Tokyo, Japan) equipped with a fluorescence unit. The intensity of fluorescence signals from the outer-layer cells of the hypocotyls was measured with Meta Imaging Series software (Metamorph; Molecular Devices, Tokyo, Japan).

Flow cytometry

The hypocotyls of the 5-day-old seedlings were cut and stained with 1% DAPI solution, and then ploidy was analyzed with a CyFlow ML counter (Partec, Görlitz, Germany) using FloMax software (Partec, Gšrlitz, Germany). Thirty hypocotyls were analyzed at a time.

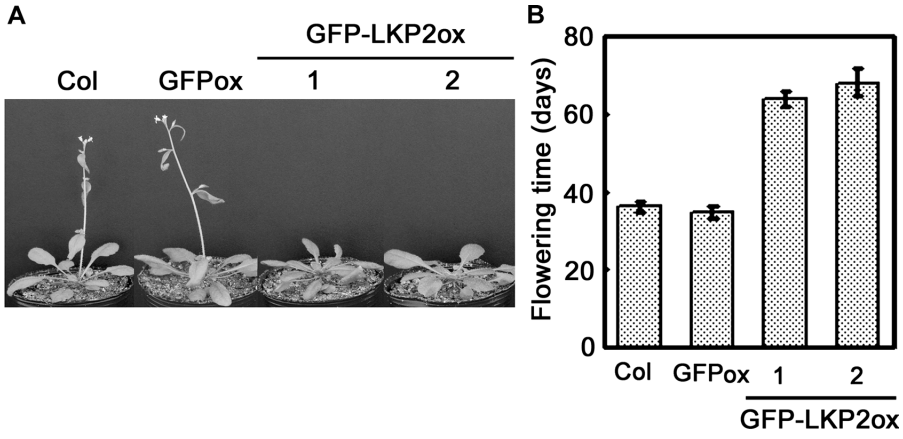

Figure 1. *LKP2* overexpression delays flowering. (A) GFP-LKP2ox and control plants grown for 5 weeks under long-day (16-h light/8-h dark) conditions. (B) Flowering time in the GFP-LKP2ox and control plants (mean±SE, $n=4$ or 5).

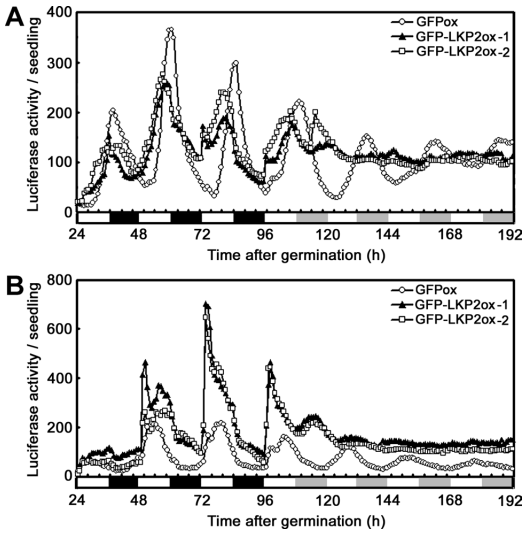

Figure 2. *LKP2* overexpression leads to a loss of circadian rhythm. (A) *pCAB2:LUC* or (B) *pCCR2:LUC* expression under 12-h light/12-h dark until 96 h after germination and then under constant light. White boxes, light phase; black boxes, dark phase; gray boxes, subjective dark phase, when the dark phase would normally have occurred.

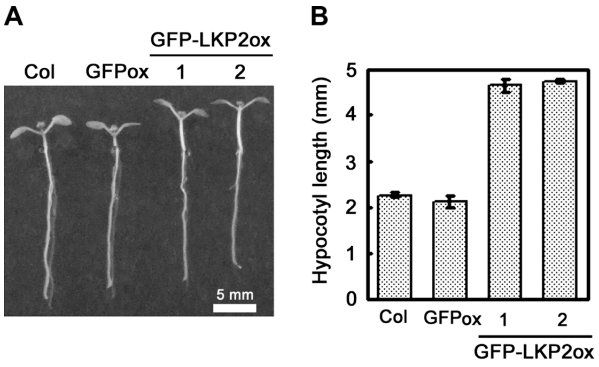

Figure 3. LKP2 overexpression enhances hypocotyl elongation. (A) GFP-LKP2ox and control seedlings grown for 5 days under constant light. (B) Hypocotyl length (mean±SE, $n=20$).

Results and discussion

GFP-LKP2-overexpressing plants exhibit late flowering, arrhythmic luciferase activity, and long hypocotyl

To study the effect of *LKP2* overexpression, we first characterized GFP-LKP2ox plants. Under long-day conditions, the GFP-LKP2ox plants flowered later than the control plants (Figure 1A, B). Both the GFP-LKP2ox plants and the control plants showed a circadian rhythm of luciferase activity, as indicated by the *pCAB2:LUC* and *pCCR2:LUC* reporters (Figure 2A, B). In contrast, under the subsequent constant-light condition, the GFP-LKP2ox plants showed a rapid loss of the circadian rhythm (Figure 2A, B). Under constant light, the hypocotyls of the GFP-LKP2ox plants were 2.1 times the length of those of the control plants (Figure 3A, B). The previous observation of these phenotypes (i.e., the alterations in flowering, circadian rhythm, and hypocotyl length) in *LKP2*-overexpressing plants (Schultz et al. 2001; Yasuhara et al. 2004) suggests that the *GFP* reporter does not affect *LKP2* function. Therefore, we further studied the relationship between *LKP2* expression and hypocotyl elongation in the GFP-LKP2ox plants.

Overexpression of LKP2 increases the length and number of hypocotyl cells

Hypocotyl elongation in *Arabidopsis* could be due to cell expansion, increases in cell number, or both (Saibo et al. 2003). To determine whether increased cell length or cell number was responsible for the hypocotyl elongation in the GFP-LKP2ox plants, we analyzed cell characteristics in 5-day-old seedlings (Figure 4). GFP-LKP2ox plants showed more cortical cells than the control plants but a similar number of epidermal cells (Figure 4A, B). This suggests that cell number in the cortex of the hypocotyls is independent of that in the epidermis. The GFP-LKP2ox plants showed longer epidermal and cortical cells than the control plants, especially in the middle of the hypocotyl (Figure 4C, D). Thus, hypocotyl

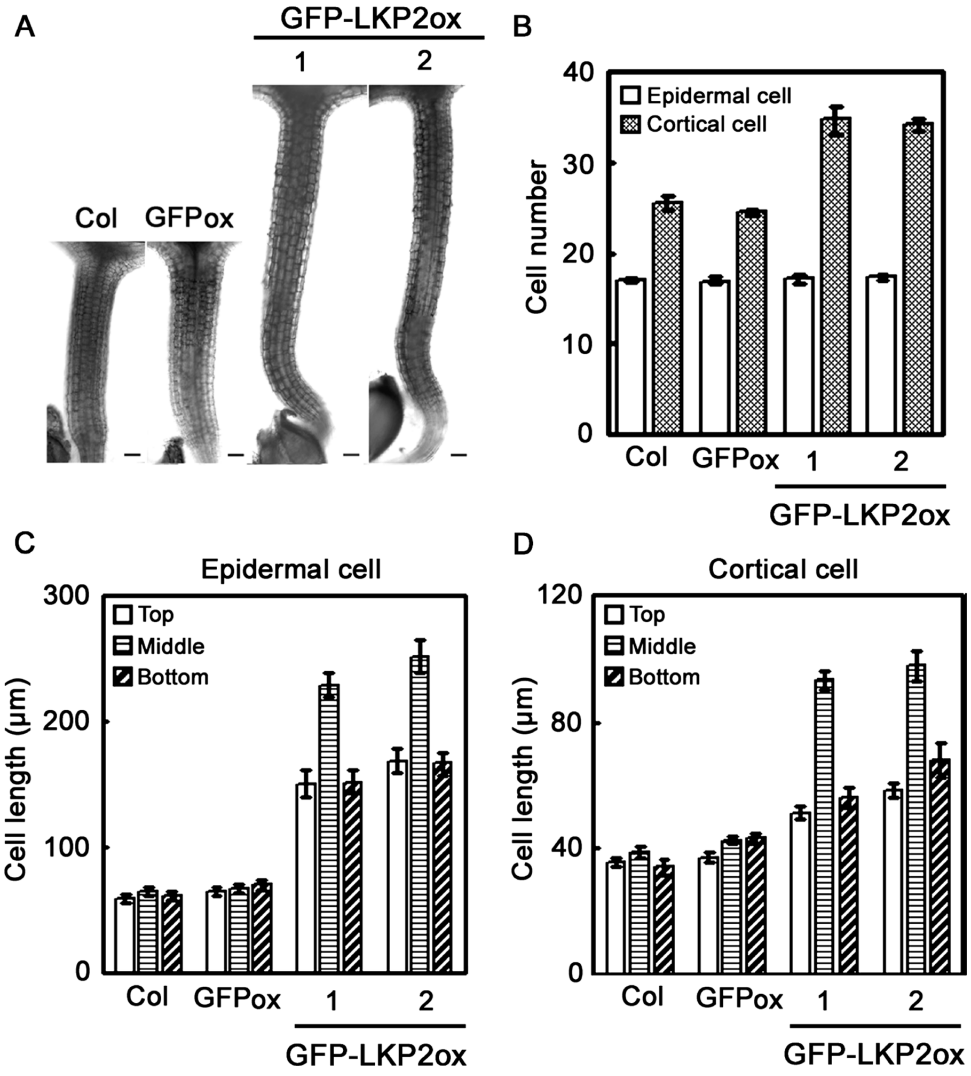

Figure 4. *LKP2* overexpression increases cell number and cell length in hypocotyls. (A) Hypocotyls of GFP-LKP2ox and control seedlings grown for 5 days under constant light. Bars=100 μm. (B) Total numbers of cells (epidermal and cortical) in individual hypocotyls (mean±SE, n=10). (C) Epidermal cell length in top, middle, and bottom parts of the hypocotyl (means±SE, n=20). (D) Cortical cell length in top, middle, and bottom parts of hypocotyl (means±SE, n=20).

elongation in the GFP-LKP2ox plants resulted from increases in both cell number and cell length.

The epidermal cells were 2.9 times as long and the cortical cells were 1.8 times as long in the GFP-LKP2ox plants as in the control plants (Figure 4C, D). Cell number increased only in the cortical cells, and the number of cortical cells in GFP-LKP2ox plants was 1.4 times that in the control plants (Figure 4B). The total hypocotyl length calculated from these changes was 2.5–2.9 times as long in GFP-LKP2ox plants as in the control plants. However, the measured hypocotyl length in GFP-LKP2ox plants was 2.1 times the length of the control (Figure 3). This difference in hypocotyl length between the result shown in Figure 3 and the result calculated from cell length and cell number may arise from our measurement of only 3 epidermal and 6 cortical cells in the 3 regions of the hypocotyl.

Cell length prominently increased in the middle region of both epidermis and cortex in GFP-LKP2ox hypocotyls. The middle region of elongation was consistent with that in non-transgenic hypocotyls that grew under constant light (Gendreau et al. 1997). Since *LKP2* was overexpressed under the control of the *35S* promoter, which acted in the cells in both the middle and other regions of the hypocotyls, the cell elongation was not cell-autonomously induced by *LKP2* overexpression (or ectopic expression).

Overexpression of LKP2 increases DNA content and nuclear size in cells

Increased cell size is often associated with an increased ploidy level in plant cells (Gendreau et al. 1997; Melaragno et al. 1993). Therefore, we analyzed the ploidy level in the GFP-LKP2ox plants. The GFP-LKP2ox plants showed fewer cells with a ploidy level of 4C (C stands for DNA/genome content) than in the

control plants and more cells with a ploidy level of 8C and 16C (Figure 5A, B). Next, we measured the DAPI signals for the nuclei in the top, middle, and bottom parts of the hypocotyls to determine which regions had an increased ploidy level. We detected stronger signals in all cells of the GFP-LKP2ox hypocotyl than in the control (Figure 6A, B).

The increase in ploidy level and DAPI signal in hypocotyl cells was consistent with the elongation of the hypocotyl cells in *LKP2*-overexpressing plants. However, the DAPI signal increased equally in the top, middle, and bottom cells (Figure 6), yet the cell elongation was greater in the middle cells in *LKP2*-overexpressing plants (Figure 4C, D). These results indicate that the increase in DAPI signal and cell elongation are not completely concurrent. Although ploidy is associated with a cell-size increase in *Arabidopsis*, it is not tightly coupled to final cell size (Beemster et al. 2002; Gendreau et al. 1998). Cells with higher ploidy exhibit endoreduplication, which is the amplification of chromosomal DNA without corresponding mitosis, although the mechanism by which endoreduplication and high ploidy levels increase cell size is not fully understood (Sugimoto-Shirasu and Roberts 2003).

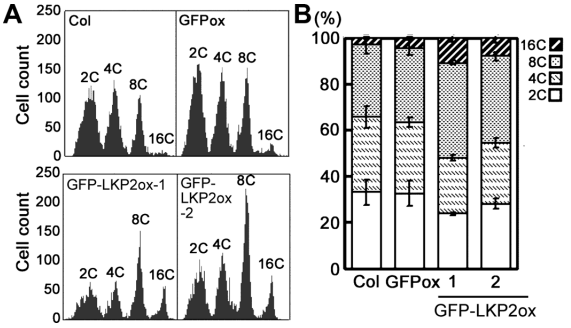

Figure 5. *LKP2* overexpression increases ploidy levels in the hypocotyl cells of GFP-LKP2ox seedlings. (A) Histograms of ploidy level in hypocotyls ($n=30$ seedlings per replicate). C stands for DNA/genome content. (B) Distribution of ploidy levels in flow cytometry data (means±SE, $n=3$ replicates).

In this study, we show that *LKP2* overexpression altered not only hypocotyl length, circadian rhythm, and flowering time, but also the DNA content in hypocotyl cells. This pleiotropic effect, where the overexpression of only 1 gene alters several phenotypic characters, suggests that (1) hypocotyl elongation, circadian clock, flowering time, and hypocotyl ploidy are governed by a common regulation mechanism, and *LKP2* overexpression influences this regulation; or (2) they are governed by different regulation mechanisms, and *LKP2* overexpression influences each of these mechanisms. We leave the task of determining which hypothesis is true to future studies.

Several phytohormones (auxins, brassinosteroids, gibberellins, and ethylene) are involved in increasing the ploidy level and cell number, and in cell expansion in *Arabidopsis* hypocotyls (Collett et al. 2000; Saibo et al. 2003; Wang et al. 1993). In addition, the expression of genes related to the biosynthesis, transport, and response of these plant hormones is regulated by the circadian clock (Harmer et al. 2000; Nozue and Maloof 2006; Schaffer et al. 2001). Therefore, the phenotype with elongated hypocotyls that results from overexpression of *LKP2* might be mediated by one or more of these phytohormones. We are now examining this hypothesis.

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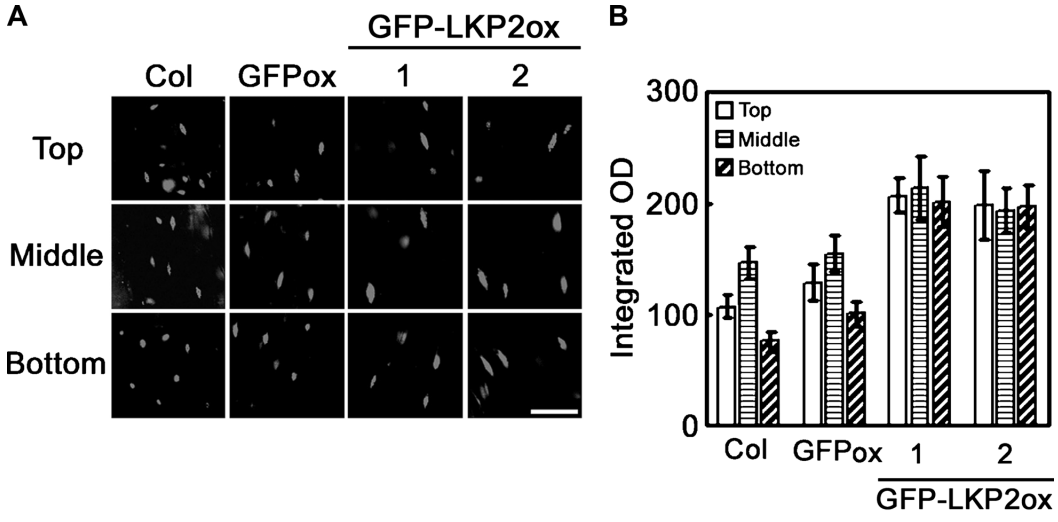

Figure 6. *LKP2* overexpression increases nucleus size. (A) Nuclear staining of hypocotyls of GFP-LKP2ox and control seedlings grown for 5 days under constant light (DAPI images). Bars=5 μm. (B) Intensity of DAPI signals per cell calculated from the DAPI images (mean optical density [OD]±SE, $n=9$–15).

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