Light-Regulated Hypocotyl Elongation Involves Proteasome-Dependent Degradation of the Microtubule Regulatory Protein WDL3 in Arabidopsis

Xiaomin Liu,1 Tao Qin,1 Qianqian Ma, Jingbo Sun, Ziqiang Liu, Ming Yuan, and Tonglin Mao2

State Key Laboratory of Plant Physiology and Biochemistry, Department of Plant Sciences, College of Biological Sciences, China Agricultural University, Beijing 100193, China

Light significantly inhibits hypocotyl cell elongation, and dark-grown seedlings exhibit elongated, etiolated hypocotyls. Microtubule regulatory proteins function as positive or negative regulators that mediate hypocotyl cell elongation by altering microtubule organization. However, it remains unclear how plants coordinate these regulators to promote hypocotyl growth in darkness and inhibit growth in the light. Here, we demonstrate that WAVE-DAMPENED 2–LIKE3 (WDL3), a microtubule regulatory protein of the WVD2/WDL family from Arabidopsis thaliana, functions in hypocotyl cell elongation and is regulated by a ubiquitin–26S proteasome–dependent pathway in response to light. WDL3 RNA interference Arabidopsis seedlings grown in the light had much longer hypocotyls than controls. Moreover, WDL3 overexpression resulted in overall shortening of hypocotyl cells and stabilization of cortical microtubules in the light. Cortical microtubule reorganization occurred slowly in cells from WDL3 RNA interference transgenic lines but was accelerated in cells from WDL3-overexpressing seedlings subjected to light treatment. More importantly, WDL3 protein was abundant in the light but was degraded through the 26S proteasome pathway in the dark. Overexpression of WDL3 inhibited etiolated hypocotyl growth in regulatory particle non-ATPase subunit1a mutant (pnp1a–4) plants but not in wild-type seedlings. Therefore, a ubiquitin–26S proteasome–dependent mechanism regulates the levels of WDL3 in response to light to modulate hypocotyl cell elongation.

INTRODUCTION

Arabidopsis thaliana seedlings exhibit different developmental patterns, depending on the ambient light. Seedlings perceive light signals via multiple photoreceptors and transduce these signals to activate downstream regulators, resulting in precise regulation of photomorphogenic developmental processes, such as termination of hypocotyl elongation, rapid root growth to anchor young plants in the soil, and opening of the cotyledon (Chen et al., 2004; Monte et al., 2007; Whitelam and Halliday, 2007). By contrast, seedlings grown in darkness become etiolated, which is associated with the presence of a rapidly elongating hypocotyl, small unopened cotyledons on an apical hook, and a short primary root (Fankhauser and Chory, 1997; Jaillais and Vert, 2012). Numerous studies have revealed that external and internal cues mediate the antagonistic effects of light and darkness on hypocotyl elongation, including plant photoreceptors, phytohormones, calcium, and transcription factors (Wang et al., 2002; Folta et al., 2003; Castillon et al., 2007; Josse et al., 2008; Tsuchida-Mayama et al., 2010; Luo et al., 2010). For example, the phytohormone ethylene has been shown to promote hypocotyl elongation in the light and suppress elongation in the dark, largely due to concomitant activation of two contrasting pathways (Shinkle and Jones, 1988; Ecker, 1995; Smalle et al., 1997; Zhong et al., 2012). Although an increasing number of upstream effectors have been identified in these signaling pathways, how plants coordinate the downstream negative and positive regulators of hypocotyl elongation in darkness and light remains an unanswered question.

Genetic and physiological studies have demonstrated that cortical microtubules play a crucial role in the regulation of cell elongation and expansion through orienting cellulose fibrils and cellulose fibril arrays to build the cell wall (Buschmann and Lloyd, 2008; Lloyd and Chan, 2008; Sedbrook and Kaoriti, 2008; Lloyd, 2011). The function of cortical microtubules is intimately linked to their organization, which can be altered by developmental and environmental cues (Dixit and Cyr, 2004). Previous studies have shown that the orientation of cortical microtubules varies with the status of hypocotyl growth (Le et al., 2005; Crowell et al., 2011). In rapidly elongating hypocotyl cells, the parallel array of cortical microtubules is predominantly transversely oriented to the hypocotyl longitudinal growth axis. By contrast, cortical microtubules are predominantly found in the oblique or longitudinal direction once the accelerative phase of cell elongation slows (Dixit and Cyr, 2004; Le et al., 2005; Li et al., 2011; Lloyd, 2011). Notably, reorganization of cortical microtubules from a transverse orientation into an oblique and longitudinal array in hypocotyl cells occurs in response to light, which also inhibits hypocotyl growth (Ueda and Matsuyama, 2000; Le et al., 2005; Sambade et al., 2012). However, the...
molecular mechanisms underlying this process are largely unclear.

Microtubule regulatory proteins regulate the organization and dynamics of microtubules (Kaloriti et al., 2007; Buschmann and Lloyd, 2008; Sedbrook and Kaloriti, 2008). An increasing number of microtubule regulatory proteins have been reported to be involved in the regulation of hypocotyl elongation through alteration of microtubule organization and dynamics. For example, decreased expression of Arabidopsis MICROTUBULE-DESTABILIZING PROTEIN40 (MDP40) results in a shorter etiolated hypocotyl phenotype, demonstrating that MDP40 functions as a negative regulator of hypocotyl elongation (Wang et al., 2012). By contrast, over-expression of MDP25 dramatically inhibits hypocotyl elongation in response to changes in cytosolic calcium levels, suggesting that MDP25 functions as a negative regulator of hypocotyl elongation in Arabidopsis (Li et al., 2011). However, how plants coordinate these downstream positive and negative regulators to mediate the different hypocotyl growth states in response to light is largely unknown.

It is well known that many fundamental cellular processes are regulated by the ubiquitin-26S proteasome system, which controls the degradation rates of numerous proteins in plants (Smalle and Vierstra, 2004; Vierstra, 2009). One such process involves the CONSTITUTIVE PHOTOMORPHOGENIC/DE-ETIOLATED/ FUSCA (COP/DFT/FUS) pathway, which serves to repress photomorphogenesis in the dark by playing a central role in the integration of far-red, red, and blue light signaling (Kwok et al., 1996). The well-known E3 ligase COP1 partitions between the nucleus and cytosol in response to darkness and light, respectively. COP1 targets positive regulators of photomorphogenesis, such as HYPOCOTYL5, LONG AFTER FAR-RED LIGHT1, and PHYTOCHROME A, for ubiquitin-mediated degradation by the 26S proteasome in order to repress photomorphogenic developmental processes, including hypocotyl elongation (Deng et al., 1992; Osterlund et al., 2000; Saijo et al., 2003; Duek et al., 2004). Lines carrying mutations in components of the ubiquitin-26S proteasome system, such as cop1 and rpm1a-4, exhibit defective etiolated hypocotyl growth (Wang et al., 2009; Chang et al., 2011). Therefore, investigation of a potential role for the 26S proteasome pathway in regulation of microtubules during hypocotyl growth is of great interest.

WDL3 belongs to the microtubule regulatory protein WAVE-DAMPENED2 (WVD2)/WVD2-LIKE (WDL) family, which contains targeting proteins for Xenopus kinesin-like protein2 in Arabidopsis (Yuen et al., 2003; Perrin et al., 2007). The conserved pentapeptide KLEEK motif was identified in an amino acid sequence from the WVD2/WDL family, which was also found in MAP70-5, a member of the microtubule regulatory protein MAP70 family (Yuen et al., 2003; Korolev et al., 2007). Constitutive expression of WVD2 results in short, thick stems and roots and inverted handedness of twisting hypocotyls and roots (Yuen et al., 2003). While MAP70-5 is involved in tracheary element development, decreased MAP70-5 resulted in reduced inflorescence stem length and diameter (Korolev et al., 2007; Pesquet et al., 2010), suggesting diverse physiological roles of KLEEK-containing proteins in plant growth and cell morphogenesis. In this study, we functionally characterized WDL3, which plays an important role in the regulation of hypocotyl cell elongation by altering the stability of cortical microtubules. More importantly, we also found that WDL3 levels are regulated by an ubiquitin-26S proteasome–dependent pathway in response to light. Our study reveals a mechanism by which the ubiquitin-26S proteasome–dependent pathway regulates microtubule organization in response to light in Arabidopsis.

RESULTS

WDL3 Functions as a Negative Regulator of Hypocotyl Cell Elongation

There are seven WVD2-like genes in the Arabidopsis genome, from WDL1 to WDL7 (Yuen et al., 2003). WDL3 expression is significantly altered in brassinosteroid phytohormone mutants based on published microarray data (Sun et al., 2010), suggesting a potential role of WDL3 in cell elongation. To analyze the function of WDL3 in Arabidopsis, WDL3 loss-of-function and WDL3-green fluorescent protein (GFP)-overexpressing seedlings were generated. Since knockdown or knockout T-DNA insertion lines of WDL3 are unavailable, we generated RNA interference (RNAi) lines to analyze the function of WDL3 in Arabidopsis. Of the fifty-five WDL3 RNAi transgenic lines that were obtained, 38 had longer hypocotyls after growth in the light. In addition, of 34 WDL3-GFP–overexpressing lines obtained, 20 exhibited shorter hypocotyls after growth in the light. Line 2 of the WDL3 RNAi transgenic lines and line 7 of the WDL3-GFP transgenic lines, which exhibited typical phenotypes, were selected for further analysis. The level of WDL3 transcription was considerably enhanced in the overexpressing line and was dramatically reduced in the RNAi line (Figure 1A). In addition, the transgenic seedlings of plants carrying WDL3 without the GFP tag and WDL3-GFP exhibited similar phenotypes (see Supplemental Figures 1A to 1C online), demonstrating that the GFP tag did not interfere with WDL3 function.

Observation of 1-week-old seedlings from the WDL3-overexpressing line revealed that the hypocotyl length was considerably reduced. By contrast, the hypocotyls were much longer in the WDL3 RNAi Arabidopsis than in wild-type plants when the seedlings were grown in the light (Figure 1B). Statistical analysis using paired Student’s t tests indicated that these differences in hypocotyl length were significant (Figure 1C). Scanning electronic microscopy revealed no major differences in the cell profiles of the hypocotyls among the wild-type, WDL3-overexpressing, and WDL3 RNAi seedlings (Figure 1D). The cell numbers in individual hypocotyl-epidermal cell files in the different seedlings were similar (20 to 22). The average widths of hypocotyl epidermal cells in wild-type, WDL3-overexpressing, and WDL3 RNAi seedlings were 27.88 ± 5.13 μm, 28.39 ± 4.92 μm, and 31.66 ± 5.71 μm, respectively (n > 500). Statistical analysis using the paired Student’s t test indicated that this difference was not significant. However, the length of hypocotyl cells was decreased in WDL3-overexpressing seedlings and significantly increased in WDL3 RNAi Arabidopsis, particularly in the middle region (Figure 1E), suggesting that WDL3 is involved in hypocotyl cell elongation. We also measured the elongation rates of hypocotyls at different times and
There was little growth of hypocotyl cells in the wild-type (Figure 1F; Sambade et al., 2012), WDL3-overexpressing, and WDL3 RNAi Arabidopsis lines (RNAi). The 18S rRNA was used as a control.

To confirm that the hypocotyl phenotype in the WDL3 RNAi line was linked to WDL3 expression levels, we randomly selected four additional RNAi lines for RT-PCR analysis. These results showed that the WDL3 expression levels corresponded with the long-hypocotyl phenotype of plants grown in the light (see Supplemental Figures 2A to 2C online). Additionally, a second WDL3 RNAi (RNAi-1) construct using another WDL3 cDNA sequence was generated. Seventeen WDL3 RNAi-1 lines exhibited a longer hypocotyl phenotype, and three independent WDL3 RNAi-1 lines were selected for further analyses. These positions along hypocotyls from plants grown in the light. There was little growth of hypocotyl cells in the wild-type (Figure 1F; Sambade et al., 2012), WDL3-overexpressing, and WDL3 RNAi Arabidopsis at days 2 and 3, but the cells were greatly elongated by day 4, especially in the middle region. In comparison to wild-type plants, the length of WDL3 RNAi hypocotyl cells was significantly increased, and the length of WDL3-overexpressing hypocotyl cells was relatively decreased at days 4 and 5 (Figure 1F). These results suggest that WDL3 plays a role in light-inhibited hypocotyl elongation.

Figure 1. WDL3 Negatively Regulates Hypocotyl Cell Elongation in the Light.

(A) RT-PCR analysis of WDL3 transcript levels in seedlings from the wild-type (WT) Columbia ecotype, WDL3-GFP transgenic (OE), and WDL3 RNAi Arabidopsis lines (RNAi). The 18S rRNA was used as a control.

(B) and (C) WDL3 RNAi seedlings have longer hypocots, whereas the hypocots are shorter in WDL3-GFP transgenic Arabidopsis (t test, **P < 0.01) grown on half-strength MS in the light for 7 d. The graph shows the average hypocotyl length measured from a minimum of 30 seedlings. Error bars represent the mean ± SD.

(D) Scanning electron microscopy images of hypocotyl epidermal cells from the wild-type, WDL3-GFP transgenic, and WDL3 RNAi Arabidopsis lines. The pink outlines show the profiles of epidermal cells in the middle of hypocots. Bar = 100 μm.

(E) Length of hypocotyl cells of the wild-type, WDL3-GFP transgenic, and WDL3 RNAi Arabidopsis lines grown in the light for 7 d. t test, *P < 0.05, **P < 0.01. Error bars represent the mean ± SD.

(F) Hypocotyl cell length of light-grown wild-type, WDL3-GFP transgenic, and WDL3 RNAi Arabidopsis seedlings was measured from days 2 to 5. The x axis shows cell number from the bottoms of the hypocotyls, and the y axis shows cell length in micrometers.
results showed that the transcription levels of WDL3 were dramatically reduced, and the hypocotyl length in 7-d-old light-grown seedlings from the WDL3 RNAi-1 lines was much longer (see Supplemental Figures 3A to 3C online). These results confirm that the longer hypocotyl phenotype of WDL3 RNAi lines grown in the light is dependent upon the expression level of WDL3. Because WDL3 belongs to the WVD2/WDL family in Arabidopsis, we detected expression of other genes in WDL3 RNAi and RNAi-1 lines. Quantitative real-time PCR analysis showed that WVD2, WDL1, WDL2, WDL4, WDL5, WDL6, and WDL7 expression in the WDL3 RNAi and RNAi-1 lines was similar to the wildtype (see Supplemental Figure 4 online), demonstrating that decreased WDL3 expression did not affect transcription levels of other genes in the WVD2/WDL family. Together, those results demonstrate that WDL3 plays a negative regulatory role in hypocotyl cell elongation in the light.

**Organization and Stability of Cortical Microtubules Are Affected in the Epidermal Cells of WDL3 Transgenic Seedlings**

Because WDL3 belongs to the microtubule regulatory protein WVD2/WDL family (Yuen et al., 2003; Perrin et al., 2007), we tested whether increasing or decreasing WDL3 expression affected the organization of cortical microtubules in hypocotyl cells. Because the transverse, oblique, and longitudinal orientation patterns of cortical microtubules are related to the elongation rate of hypocotyl epidermal cells, hypocotyls are frequently used to explore the mechanisms that underlie cell elongation and cortical microtubule organization (Le et al., 2005; Crowell et al., 2011; Motose et al., 2011). Therefore, we analyzed cortical microtubules in epidermal cells from the hypocotyl middle region of wild-type, WDL3-overexpressing, and WDL3 RNAi Arabidopsis seedlings carrying tubulin tagged with GFP. Cells were examined at days 4 and 5 of exposure to light. The parallel arrays of cortical microtubules were generally transversely and obliquely oriented relative to the longitudinal hypocotyl growth axis in epidermal cells from wild-type hypocotyls (Figures 2A and 2D). In comparison, random, oblique, or longitudinal cortical microtubules were observed in most of the WDL3-overexpressing hypocotyl cells, while dominantly transverse cortical microtubule arrays were detected in the WDL3 RNAi Arabidopsis cells at day 4 (Figures 2B to 2D). Cortical microtubules were mostly oriented in a longitudinal manner in the wild-type and WDL3-overexpressing hypocotyl cells at day 5 (Figures 2E, 2F, and 2H), while oblique and transverse cortical microtubules were observed in most of the WDL3 RNAi Arabidopsis at day 5 (Figures 2G and 2H). The altered microtubule arrays observed in WDL3 RNAi seedlings were consistent with the significant increase in hypocotyl cell elongation caused by reduced expression of WDL3.

In order to further characterize the effect of WDL3 on cortical microtubules, the microtubule-disrupting drug oryzalin was applied to epidermal hypocotyl cells from wild-type, WDL3-overexpressing, and WDL3 RNAi seedlings. To quantify the effect of oryzalin on the stability of cortical microtubules, we estimated the density of cortical microtubules in hypocotyl epidermal cells.

**Figure 2. The Cortical Microtubule Array Is Greatly Altered in Hypocotyl Epidermal Cells of WDL3 Transgenic Seedlings.**

(A) to (C) and (E) to (G) Cortical microtubules in epidermal cells from the middle region of the hypocotyl of wild-type (WT), WDL3-GFP transgenic (OE), and WDL3 RNAi (RNAi) seedlings with a GFP-tubulin background were observed by confocal microscopy after growth in the light for 4 or 5 d. Bar in (G) = 10 μm. (D) and (H) Frequency of different microtubule orientation patterns in light-grown hypocotyl epidermal cells from wild-type, WDL3-GFP transgenic, and WDL3 RNAi seedlings (n > 150 cells).

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A paired Student’s t test was used to identify significant differences. Our results revealed that cortical microtubules were similar in density before oryzalin treatment in the epidermal cells of wild-type, WDL3-overexpressing, and WDL3 RNAi seedlings (Figure 3A). However, the number of cortical microtubules in epidermal cells of wild-type, WDL3-overexpressing, and WDL3 RNAi seedlings was significantly different after drug treatment (Figure 3F). Microtubules were disrupted in epidermal cells from the WDL3 RNAi line after treatment with 5 µM oryzalin for 5 min (Figures 3B and 3F), whereas microtubules in wild-type and WDL3-overexpressing cells were largely unaffected. Increases in the concentration and duration of oryzalin treatment resulted in disruption of the majority of cortical microtubules in both wild-type and WDL3 RNAi Arabidopsis cells. However, cortical microtubules remained relatively unaffected in WDL3-overexpressing cells at doses of up to 10 µM oryzalin (Figures 3C, 3D, and 3F). In addition, although cortical microtubules mostly recovered in cells from wild-type and WDL3-overexpressing Arabidopsis hypocotyls after oryzalin washout, many cortical microtubules remained disrupted in cells from WDL3 RNAi Arabidopsis hypocotyls (Figures 3E and 3F). Thus, microtubules were more sensitive to oryzalin treatment in WDL3 RNAi cells and less sensitive when expression of WDL3 was increased. These results confirm that WDL3 functions as a microtubule stabilizer. Microtubule dynamics were analyzed using confocal time-lapse imaging. Microtubules with clearly visible leading plus ends (identified by growth rates) were selected for measurement in wild-type, WDL3-overexpressing, and WDL3 RNAi cells (see Supplemental Table 1 online). The results show that microtubule dynamics were altered in WDL3-overexpressing and RNAi hypocotyl epidermal cells. The catastrophe frequency (frequency of transitions from growth or pause to shortening) of individual microtubules was significantly lower in WDL3-overexpressing cells (0.006 s⁻¹ for catastrophe) and higher in WDL3 RNAi cells (0.042 s⁻¹ for catastrophe) compared with wild-type cells (0.027 s⁻¹ for catastrophe), suggesting that individual microtubules are more prone to growth when expression of WDL3 is increased but are more likely to shrink in the absence of WDL3.

**Figure 3.** Cortical Microtubules are Hypersensitive to Oryzalin Treatment in WDL3 RNAi Cells but More Resistant in WDL3 Transgenic Cells. (A) to (D) Cortical microtubules were observed in epidermal cells from light-grown hypocotyls in wild-type (WT), WDL3 transgenic (OE), and WDL3 RNAi (RNAi) seedlings after treatment with 0 µM oryzalin (A), 5 µM oryzalin for 5 min (B), 10 µM oryzalin for 10 min (C), and 10 µM oryzalin for 30 min (D). (E) After the treatment in (D), oryzalin was washed off, and cortical microtubules were observed after 2 h. Bar = 10 µm. (F) Quantification of cortical microtubules in hypocotyl epidermal cells of wild-type, WDL3 transgenic, and WDL3 RNAi seedlings using ImageJ software (n > 28 cells for each sample). The y axis represents the number of cortical microtubules that crossed a fixed line (~10 µm) perpendicular to the orientation of the majority of cortical microtubules in the cell. Student’s t tests were performed to compare the numbers of cortical microtubules in hypocotyl epidermal cells from WDL3 transgenic and WDL3 RNAi lines with that of wild-type plants under the same conditions. **P < 0.01 and *P < 0.05. Error bars represent the mean ± so. [See online article for color version of this figure.]

**WDL3 Levels Are Downregulated by 26S Proteasome–Dependent Degradation in the Dark**

Unlike plants grown in the light, the lengths of etiolated hypocotyls from wild-type, WDL3-overexpressing, and WDL3 RNAi seedlings were not obviously different (see Supplemental Figures 5A and 5B online), suggesting that WDL3 function is inhibited in the dark. To better understand this phenomenon, levels of the WDL3 transcript were assessed to determine whether WDL3 was expressed in the absence of light. A construct was made in which the β-glucuronidase (GUS) reporter gene was placed under control of the −1.7-kb WDL3 promoter. This construct (P<sub>WDL3</sub>:GUS) was introduced into wild-type plants using Agrobacterium tumefaciens–mediated transformation. Thirty-seven independent transgenic lines were stained for GUS activity. GUS staining revealed that WDL3 was expressed in a majority of the tissues and organs of Arabidopsis (see Supplemental Figures 6A to 6H online), including the hypocotyls of seedlings grown in the light or in the dark (Figure 4A). To verify this result, RNA was purified from the hypocotyls of light- and dark-grown wild-type seedlings. RT-PCR revealed that the WDL3 transcript was abundant in both light-grown and etiolated hypocotyls (Figure 4B), demonstrating that transcription of WDL3 does not appear to be regulated by the presence or absence of light. Next, WDL3 protein levels were analyzed using WDL3-GFP transgenic seedlings. Confocal microscopy showed that WDL3-GFP filaments were clearly detected in the hypocotyl epidermal cells of light-grown seedlings but not in etiolated hypocotyl epidermal cells of WDL3-GFP transgenic lines (Figure 4C). Protein gel blots using an anti-GFP antibody
confirmed this result (Figure 4D), demonstrating that WDL3-GFP levels were more abundant in the light and less abundant in the dark.

To analyze the potential molecular mechanism of WDL3 degradation in the dark, seedlings were treated with MG132 and MG115, two 26S proteasome inhibitors (Lee and Goldberg, 1998; Planchais et al., 2000). Confocal microscopy revealed that WDL3-GFP fluorescence was recovered in etiolated hypocotyls from WDL3-GFP transgenic seedlings treated with MG132 and MG115 (Figures 5A to 5C). By contrast, mock treatment with ethanol (Figure 5D) or with other general protease inhibitors (leupeptin and phenylmethylsulfonyl fluoride) had little or no effect on the degradation of WDL3-GFP (Figures 5E and 5F). Thus, the degradation of WDL3 is prevented by treatment with proteasome-specific inhibitors, suggesting that the proteasome is largely responsible for the degradation of WDL3 in the absence of light.

Next, we examined WDL3 protein levels during dark–light transitions. WDL3-GFP transgenic seedlings were grown in darkness for 6 d and then transferred to continuous light for 4, 8, 12, 16, 20, and 24 h. Protein gel blots of protein extracts from these seedlings revealed that the abundance of WDL3 was significantly increased after 4 h and 8 h of light. Seedlings grown in continuous light were included as a positive control, and the WDL3 levels in these plants were significantly higher than that of plants subjected to the dark (Figure 5G). Treatment with cycloheximide,
an inhibitor of cytoplasmic protein synthesis, was then used to determine whether protein degradation or protein synthesis represents the key regulatory step that modulates differential abundance of WDL3 in light and darkness. Six-day-old WDL3-GFP transgenic seedlings grown in continuous light were transferred to darkness and cultured in the presence of 200 μM cycloheximide. The cultures were then treated in darkness for 0, 12, and 24 h, and the levels of WDL3-GFP were measured. Samples maintained in the light served as controls. In the absence of cycloheximide, the amount of WDL3 was decreased when seedlings were transferred to darkness. The amount of WDL3 decreased within 24 h under both light and dark conditions in the presence of cycloheximide; however, the rate of decrease was significantly greater at 12 h after transfer to darkness (Figure 5H). Because cycloheximide effectively blocks new protein synthesis, the reduced levels of WDL3 must therefore be due to the degradation of preexisting protein. Together, these results suggest that WDL3 is degraded by a 26S proteasome-dependent pathway in the absence of light.

Because our pharmacological results suggested that degradation of the WDL3 protein was mediated by the 26S proteasome, we next tested whether overexpression of WDL3 could inhibit etiolated hypocotyl growth in a 26S proteasome mutant background. The RPN1 protein, encoded by RPN1a, is a non-ATPase subunit of the regulatory particles of the 26S proteasome complex in Arabidopsis and plays a crucial role in the functioning of the 26S proteasome (Rosenzweig et al., 2008). Therefore, we crossed the WDL3-GFP transgenic plant with rpn1a-4, a null mutant of RPN1a (Wang et al., 2009). Fifteen resulting WDL3-GFP:rpn1a-4 lines exhibited a shorter etiolated hypocotyl phenotype, and line 2, which exhibited the typical phenotype, was selected for further analyses. Confocal images revealed that WDL3-GFP was present in filamentous structures in etiolated hypocotyl epidermal cells from homozygous WDL3-GFP:rpn1a-4 mutant seedlings but not WDL3 transgenic wild-type seedlings (Figures 6A and 6B). These filamentous structures containing WDL3-GFP could be disrupted by treatment with oryzalin (Figure 6C) and recovered after oryzalin washout (Figure 6D), demonstrating that WDL3 is also capable of binding cortical microtubules in the dark. Observation of 5-d-old etiolated seedlings from rpn1a-4 and WDL3-GFP:rpn1a-4 lines revealed that overexpression of WDL3-GFP substantially decreased...
the etiolated hypocotyl length of the rpn1a-4 mutant (Figure 6E). Scanning electronic microscopy revealed no major differences in the cell profiles of hypocotyls between rpn1a-4 and WDL3-GFP; rpn1a-4 seedlings (Figure 6F). The cell numbers in individual hypocotyl-epidermal cell files in different seedlings were similar (~20 to 22). The average widths of hypocotyl epidermal cells in rpn1a-4 and WDL3-GFP; rpn1a-4 seedlings were 21.48 ± 3.52 μm and 22.31 ± 3.81 μm (n > 300), respectively. Statistical analysis using paired Student’s t test indicated that this difference was not significant. However, the length of hypocotyl cells was significantly decreased in WDL3-GFP transgenic rpn1a-4 mutants (Figure 6G), which is consistent with a negative role of WDL3 in hypocotyl cell elongation.

**Alteration of WDL3 Expression Levels Affects Cortical Microtubule Reorganization in Response to Light**

Since light induces cortical microtubule reorganization from transverse arrays into oblique and longitudinal patterns in hypocotyl cells after exposure of etiolated plants to light (Ueda and Matsuyama, 2000; Le et al., 2005), we investigated the effects of WDL3 on this transition in elongating epidermal cells. Wild-type, WDL3-overexpressing, and WDL3 RNAi seedlings were grown for 3 d in the dark on half-strength Murashige and Skoog (MS) medium, followed by exposure to white light for 0, 30, or 60 min. First, we assessed WDL3-GFP protein levels after transfer from darkness to light. Protein gel blots showed that WDL3-GFP levels were greatly increased after treatment with light for 30 and 60 min (Figure 7A). Confocal microscopy revealed that, after 3 d of growth in the dark, parallel arrays of cortical microtubules were transversely oriented relative to the hypocotyl growth axis in epidermal cells from the etiolated hypocotyl upper regions from wild-type, WDL3-overexpressing, and WDL3 RNAi seedlings (Figures 7B, 7E, 7H, and 7K). After treatment with light for 30 min, transverse and oblique cortical microtubule arrays were dominantly observed in hypocotyl cells of the wild-type and WDL3 RNAi seedlings (Figures 7C, 7I, and 7K) but not in the WDL3-overexpressing *Arabidopsis* cells. The overexpressing cells predominately exhibited longitudinal

![Figure 7. Light-Induced Reorganization of Cortical Microtubules Occurs More Quickly in WDL3 Transgenic Arabidopsis Cells and More Slowly in WDL3 RNAi Arabidopsis Cells.](image-url)

(A) Protein gel blot of WDL3-GFP seedlings grown in the dark for 3 d, then transferred to light for 0 (lane 2), 30 (lane 3), and 60 min (lane 4). Wild-type (WT) seedlings grown in the light served as a control (lane 1). A nonspecific band appearing on the blot serves as a loading control.

(B) to (J) Etiolated hypocotyls from wild-type (B) to (D), WDL3 transgenic (E) to (G), and WDL3 RNAi (H) to (J) seedlings with a GFP-tubulin background were grown on half-strength MS medium until day 3 and then transferred to the light for 0, 30, and 60 min. Cortical microtubules were observed in epidermal cells from the upper regions of the hypocotyls. (B), (E), and (H) are without light treatment; (C), (F), and (I) are treated with light for 30 min; (D), (G), and (J) are treated with light for 60 min. Bar in (J) = 10 μm.

(K) Frequency of microtubule orientation patterns in the epidermal cells from the upper regions of the hypocotyls of the wild-type, WDL3 transgenic, and WDL3 RNAi lines (n > 104 cells).

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microtubule arrays (Figures 7F and 7K). Increasing the duration of treatment induced the formation of longitudinal cortical microtubule arrays in the wild-type and WDL3-overexpressing cells (Figures 7D, 7G, and 7K) but not in the WDL3 RNAi line (Figures 7J and 7K). These results indicate that cortical microtubule reorganization was hindered in WDL3 RNAi cells but accelerated in WDL3-overexpressing cells in response to light treatment, demonstrating that WDL3 is necessary for light-regulated cortical microtubule reorganization.

**Recombinant WDL3 Binds to, Bundles, and Stabilizes Microtubules in Vitro**

Given that WDL3 is required for cortical microtubule stability in hypocotyls and normal hypocotyl cell elongation, we investigated the molecular basis for WDL3 regulation of microtubules in vitro. We cloned the full-length CODING SEQUENCE (CDS) into the pGEX-4T vector and transformed the plasmid into *Escherichia coli*. The resulting glutathione-S-transferase (GST)-tagged WDL3 fusion protein was then purified using a glutathione-one sepharose 4B resin column. SDS-PAGE revealed that GST-WDL3 was present in two major bands, the lower of which was predicted to be a degradation product based on immunoblots using an anti-GST antibody (see Supplemental Figures 7A and 7B online).

In order to determine whether WDL3 binds to microtubules, a cosedimentation assay was used to analyze binding of WDL3 to paclitaxel-stabilized microtubules. GST-WDL3 (4 μM) was incubated with preformed 5 μM paclitaxel-stabilized microtubules at room temperature for 20 min, followed by centrifugation. SDS-PAGE analysis revealed that GST-WDL3, but not GST alone, bound and cosedimented with the microtubules (Figure 8A). Then, the subcellular localization of WDL3 was investigated. A construct expressing WDL3 fused with a C-terminal GFP tag under control of the 35S promoter was generated and transiently introduced into Arabidopsis cells. Confocal microscopy revealed that WDL3-GFP formed filamentous structures in pavement cells (Figure 8B). The filaments were mostly intact in the presence of latrunculin A (LatA), a reagent that depolymerizes actin filaments (Figure 8C), but were disrupted by treatment with the microtubule-disrupting reagent oryzalin (Figure 8D). After washout of oryzalin, the filamentous WDL3-GFP structures recovered (Figure 8E). To confirm this result, WDL3-GFP and MBD-mCherry (mCherry-tagged microtubule binding domain of MAP4) were transiently coexpressed in *Arabidopsis* pavement cells. Confocal microscopy showed that the green fluorescent signal of WDL3-GFP overlapped with the red fluorescent signal of MBD-mCherry, confirming that WDL3 localized to microtubules (Figures 8F to 8H). Co-localization was analyzed by plotting the signal intensities of WDL3-GFP and MBD-mCherry using ImageJ software (Figures 8I and 8J). Filamentous structures that could be disrupted by oryzalin, but not LatA, were also observed in hypocotyl epidermal cells of *Arabidopsis* seedlings stably expressing WDL3-GFP (see Supplemental Figures 8A to 8C online). By contrast, actin filaments decorated with stably expressed FABD2-GFP (GFP tagged the second actin binding domain of *Arabidopsis* fimbrin) were disrupted by treatment with LatA (see Supplemental Figures 8D and 8E online). These data demonstrate that WDL3 colocalizes with microtubules in vitro and in cells.

**Effects of WDL3 on Microtubules**

Microtubules were polymerized from rhodamine-labeled tubulins exhibited single filament patterns in the absence of WDL3, as observed using confocal microscopy (Figure 9A) and negative-staining electron microscopy (Figure 9E). However, addition of GST-WDL3 or WDL3 (without the GST tag) resulted in formation of large microtubule bundles (Figures 9B, 9C, 9F, and 9G). To strip WDL3 from the microtubules, 200 mM NaCl was added to the reaction. Following the addition of NaCl, WDL3-induced microtubule bundles dispersed into single filaments (Figure 9D). To further confirm microtubule bundling by WDL3, a low-speed cosedimentation
assay was performed. *Arabidopsis* microtubule-bundling protein MAP65-1 was used as a control (Mao et al., 2005). Tubulin (20 μM) was assembled at 35°C for 30 min in the presence or absence of 8 μM WDL3 or 6 μM MAP65-1 and centrifuged at 5900 g. Microtubules alone did not sediment following low speed centrifugation (Figure 9H, lanes 1 and 2). However, the majority of microtubules appeared in the low-speed pellets when incubated with WDL3 or the MAP65-1 fusion protein (Figures 9H, lanes 3 to 6, and 9I). These data indicate that WDL3 plays a role in microtubule bundling.

**Figure 9.** WDL3 Bundles and Stabilizes Microtubules in Vitro.

(A) to (H) WDL3 induces formation of microtubule (MT) bundles in vitro. Fluorescent images are shown in (A) to (D), and electron micrographs are shown in (E) to (G). Bar in (G) = 500 nm.

(A) and (E) Microtubules polymerized in the absence of WDL3.

(B) and (F) Microtubule bundles induced by GST-WDL3.

(C) and (G) Microtubule bundles induced by WDL3.

(D) Microtubule bundles induced by WDL3 were sensitive to NaCl. Single microtubules were observed after treatment with 200 mM NaCl.

(H) Low-speed cosedimentation of WDL3 with microtubules. Tubulin (20 μM) was assembled with or without 8 μM WDL3 or 6 μM MAP65-1 fusion proteins for 30 min and centrifuged at low speed. Lane 1, microtubule pellet; lane 2, microtubule supernatant; lane 3, microtubule pellet in the presence of WDL3; lane 4, microtubule supernatant in the presence of WDL3; lane 5, microtubule pellet in the presence of MAP65-1; lane 6, microtubule supernatant in the presence of MAP65-1.

(I) Statistical analysis for (H). The resulting gels were scanned to determine the distribution of tubules in the pellets in the presence of 0, 8 μM WDL3, or 6 μM MAP65-1, respectively. Error bars represent ± SD (n = 3). WDL3 stabilized microtubules against cold and dilution induced depolymerization.

(J) to (R) Images of microtubules polymerized from rhodamine-labeled tubulin (20 μM) incubated in the presence or absence of 3 μM WDL3 or MAP65-1 protein for 30 min. Samples from (J) to (L) were subjected to 10°C for 30 min ([M] to [O]) or diluted with a solution containing WDL3 or MAP65-1 in 50X PEM buffer ([P] to [R]). Bar in (D) and (R) = 10 μm.
Next, we tested whether WDL3 could protect microtubules against cold-induced and dilution-induced depolymerization. MAP65-1, which has been shown to stabilize microtubules under these conditions (Mao et al., 2005), was used as a control. Rhodamine-labeled tubulin (20 μM) was incubated in the presence and absence of WDL3 (3 μM) or MAP65-1 (3 μM) to allow tubulin polymerization (Figures 9J to 9L). The solutions were then incubated at 10°C for 30 min (Figures 9M to 9O) or diluted with 50 x prewarmed buffer and incubated at 35°C for 60 min (Figures 9P to 9Q) prior to fixation. After fixation, the samples were observed by confocal microscopy. The results showed that microtubule filaments in the absence of WDL3 were fully disassembled after cold and dilution treatments (Figures 9M and 9P). However, many microtubules persisted in the presence of WDL3 (Figures 9N and 9Q) and MAP65-1 (Figures 9O and 9R) after the cold and dilution treatments. These results indicate that WDL3 is capable of stabilizing microtubules against low-temperature and dilution induced depolymerization.

DISCUSSION

In this study, we demonstrate that the ubiquitin-26S proteasome degradation system is involved in modulation of the microtubule regulatory protein WDL3. Together, these proteins comprise a regulatory mechanism that mediates the antagonistic effects of light and darkness on hypocotyl cell elongation.

Stabilization or Destabilization of Cortical Microtubules Leads to Altered Hypocotyl Growth

The orientation of cortical microtubules is widely accepted to be closely associated with hypocotyl cell elongation (Le et al., 2005). Microtubule regulatory proteins affect microtubule stability in order to remodel microtubule arrays in plant cells. However, it remains unclear how microtubule stabilizers and destabilizers differentially contribute to the status of hypocotyl growth. Genetic and pharmacological evidence has shown that destabilization of cortical microtubules results in inhibition of hypocotyl cell elongation (Le et al., 2005; Li et al., 2011). For example, overexpression of MAP18 and MDP25 results in disordered cortical microtubule orientation and destabilizes microtubules in hypocotyl epidermal cells, inhibiting hypocotyl elongation (Wang et al., 2007; Li et al., 2011). Arabidopsis double mutants for microtubule-stabilizing protein MAP65-1 and MAP65-2 also exhibit defective hypocotyl growth (Smertenko et al., 2004; Mao et al., 2005; Gaillard et al., 2008; Li et al., 2009; Lucas et al., 2011). However, our findings support the possibility that overstabilization of cortical microtubules also alters microtubule organization and consequently inhibits hypocotyl cell elongation. Together, these studies suggest that alteration of cortical microtubule stability, either by overstabilization or destabilization, can induce abnormal hypocotyl cell elongation.

Although WDL3 exhibited microtubule-bundling activity in vitro, large microtubule bundles were not observed in WDL3-overexpressing seedlings. This phenomenon could be due to (1) relatively low expression of WDL3 or (2) relatively weaker WDL3 microtubule-bundling activity, as suggested by the low-speed cosedimentation assay compared with MAP65-1 (Figures 9H and 9I). Overexpression of WDL3 results in the formation of short hypocotyls by stabilizing cortical microtubules; however, the molecular mechanisms underlying this regulatory pathway are complicated. It remains unclear whether the stabilizing activity of WDL3 is transient or prolonged in nature with respect to inhibition of hypocotyl growth. Analysis of transcription demonstrated that stabilizers and destabilizers, such as MAP18 and WDL3, are expressed in hypocotyls from seedlings grown in the light (Wang et al., 2007; this study). Thus, how plants specifically coordinate these regulators in the same cell in order to favor inhibition of hypocotyl elongation in the light will be a subject for future studies.

Multiple Layers of Regulation Are Involved in Microtubule-Mediated Hypocotyl Elongation in Response to Light

Previous studies and our results indicate that organization of cortical microtubules is altered when etiolated hypocotyls are treated with light (Figure 7; Le et al., 2005). In addition, cortical microtubules in the hypocotyl epidermal cells of light-grown seedlings are more stable than those of seedlings grown in darkness (see Supplemental Figure 9 online; Sambade et al., 2012). Thus, precise regulation of reorganization and stabilization of cortical microtubules is required for hypocotyl growth in response to light. Importantly, these processes can be regulated by specific microtubule regulatory proteins.

Many microtubule regulatory proteins play positive or negative roles in hypocotyl cell elongation by altering the stability and organization of cortical microtubules. Plants must properly coordinate multiple pathways to modulate the activity of these...
regulated by light in hypocotyl cells. Furthermore, Ca$^{2+}$ regulates the activity of MDP25 on cortical microtubules, and overexpression of MDP25 has been shown to inhibit hypocotyl elongation by destabilizing cortical microtubules (Johnson et al., 1995; Folet et al., 2003; Li et al., 2011). Thus, MDP25 regulates hypocotyl elongation in a Ca$^{2+}$-dependent manner in response to light. A second mechanism that influences the activity of microtubule regulatory proteins during hypocotyl elongation involves transcript regulation. For example, SPIRAL1 (SPR1) transcripts were detected in the cells of etiolated hypocotyls, but not light-grown hypocotyl cells, and the hypocotyls of spr1 mutant plants display helical growth defects when grown in the dark (Nakajima et al., 2004, 2006). Therefore, light affects SPR1 expression, which modulates SPR1-mediated regulation of hypocotyl growth in response to light. A third mechanism that may be involved in mediating microtubule regulatory protein function during hypocotyl elongation is posttranslational regulation. The Arabidopsis double map65-1 map65-2 mutant exhibited defective hypocotyl growth, suggesting a role for these proteins in the regulation of microtubule-dependent hypocotyl elongation (Lucas et al., 2011). Furthermore, Nt-MAP65-1, the tobacco (Nicotiana tabacum) homolog of MAP65-1 and MAP65-2, is phosphorylated and its microtubule-bundling activity is regulated by kinases of the tobacco mitogen-activated protein kinase cascade, specifically NRK1/NTF6 (Hussey et al., 2002; Sasabe et al., 2006). In addition, phosphatidic acid, a product of phospholipase D, directly binds MAP65-1 and promotes its microtubule-polymerizing and bundling activities to stabilize cortical microtubules (Zhang et al., 2012). Thus, phosphorylation and phosphatidic acid–mediated posttranslational regulation may play key roles in MAP65-mediated hypocotyl growth.

Our study has revealed that the proteasome-dependent protein degradation pathway is also important for posttranslational regulation of the abundance of microtubule regulatory proteins in order to modulate hypocotyl growth in light and darkness. Plants may regulate the levels of proteins that regulate hypocotyl elongation in order to alter the organization and stabilization of cortical microtubules to facilitate hypocotyl growth in the dark or inhibit hypocotyl elongation in the light. In addition to these pathways, future studies should explore whether other posttranslational modifications are involved in modulating the functions of microtubule regulatory proteins, and thereby hypocotyl cell elongation, in response to the antagonistic effects of light and darkness.

The Ubiquitin-26S Proteasome System Is Involved in the Regulation of Hypocotyl Elongation in Response to Light

The ubiquitin-26S proteasome system regulates the accumulation of many upstream positive and negative regulators to promote optimal Arabidopsis hypocotyl growth in the dark and the light (Osterlund et al., 2000; Catalá et al., 2011; Chang et al., 2011). A few studies have shown that the 26S proteasomal degradation pathway is involved in regulating plant microtubules in response to environmental and developmental cues. Arabidopsis SPR1, a microtubule-stabilizing protein, is degraded by the 26S proteasome in order to promote microtubule disassembly during acclimation of plant cells to salt stress (Wang et al., 2011), suggesting that proteasome-dependent degradation of microtubule regulatory proteins is also required for microtubule-based functions.

Previous studies of rpn10-1 and rpn1a-4 mutants have shown that abnormal functioning of the 26S proteasome system inhibits etiolated hypocotyl growth (Wang et al., 2009). In addition, cortical microtubules are more stable in the rpn10-1 mutant background (Wang et al., 2011), suggesting that 26S proteasome–regulated hypocotyl growth requires destabilization of cortical microtubules. Promotion of microtubule destabilization may be achieved through controlled degradation of microtubule stabilizers, such as WDL3. In this study, although we failed to generate an anti-WDL3 antibody capable of detecting endogenous levels of the native WDL3 protein, we showed that genetic and pharmacological inhibition of etiolated hypocotyl elongation was observed in the 26S proteasome–mutant rpn1a–4. These data suggest that WDL3 plays a role in 26S proteasome–mediated hypocotyl growth in response to light. In addition, a previous study showed that constitutive expression of WVD2 resulted in defective growth of etiolated hypocotyls, demonstrating that the WVD2 protein functions in the dark (Yuen et al., 2003). Filament structures of three other members of the WVD2/WDL family (WDL4-GFP, WDL5-mCherry, and WDL6-mCherry) were detected in the light- and dark-grown hypocotyl cells of transgenic Arabidopsis (see Supplemental Figures 10A to 10C online). This evidence suggests that a specific function of WDL3 involves 26S proteasome–mediated hypocotyl cell elongation in response to light.

Characterization of WDL3 provides strong evidence for a role in the ubiquitin-26S proteasome degradation system in the regulation of microtubules in plant cells in order to coordinate antagonistic effects on hypocotyl growth in light and darkness. Based on these observations, we propose the following model describing the function of WDL3 in light-inhibited hypocotyl cell elongation (Figure 10). In the dark, we propose that the hypocotyl-elongating inhibiting activity of WDL3 is inhibited due to degradation of WDL3 by the ubiquitin-26S proteasome system. By contrast, WDL3 is stabilized in the light, resulting in WDL3–mediated stabilization of cortical microtubules, which promotes longitudinal orientation of the microtubules and inhibits hypocotyl cell elongation.

METHODS

Plant Materials and Growth Conditions

All Arabidopsis thaliana plants and materials used in this study were in the Columbia-0 ecotype background. Seeds were sterilized and placed on half-strength MS medium (Sigma-Aldrich) containing 0.8% agar and 1% Suc. For hypocotyl measurement, plates were placed at 22°C in the light for 5 or 7 d after stratification at 4°C for 3 d. Mutant rpn1a–4 plants and 3SS:Tubulin6A-GFP transgenic plants have previously been described (Wang et al., 2007; Wang et al., 2011).
Isolation of WDL3 cDNA Clones from Arabidopsis

The full-length WDL3 cDNA sequence was amplified by RT-PCR. Primers used to amplify WDL3 were 5'-GGATCCAGGATCTCTAGGACAAGG-3' and 5'-GTCGACATTGAGAAGCAAGCTC-3'. GST-tagged fusion proteins were expressed and purified according to the manufacturer's protocols. Protein concentration was determined using a Bio-Rad protein assay kit. Protein samples were analyzed by SDS-PAGE.

Microtubule Cosedimentation Assay

Porcine brain tubulins were purified using a previously published method by Castoldi and Popov (2003). These purified tubulins were used for cosedimentation assays. Tubulin assembly and cosedimentation of microtubules with the fusion proteins were performed as described by Mao et al. (2005) and Li et al. (2011). The purified proteins were centrifuged at 150,000g at 4°C for 20 min before use. Prepolymerized, paclitaxel-stabilized microtubules (5 μM) were incubated with 4 μM WDL3 fusion proteins in PEM buffer (1 mM MgCl2, 1 mM EGTA, and 100 mM PIPES-KOH, pH 6.9) plus 20 μM GTP, at room temperature for 20 min. After centrifugation at 100,000g for 20 min, the supernatant and pellets were subjected to SDS-PAGE.

Low-Temperature and Dilution Assays

Purified tubulin was conjugated to 5- and 6-carboxyethylrhodamine succinimidyl ester (NHS-rhodamine, as previously reported (Hyman, 1991). NHS-rhodamine-labeled tubulin underwent an additional round of assembly/disassembly with 30% (v/v) glycerol prior to storage in liquid nitrogen.

GST-WDL3 protein or GST-MAP65-1 protein (3 μM) was added to 20 μM rhodamine-labeled tubulin in PEM buffer containing 1 mM GTP. After tubulin assembly at 35°C for 40 min, the temperature was immediately decreased to 10°C and maintained for 30 min for low-temperature experiments. For dilution treatments, the assembled tubulin samples described above were diluted with 50x prewarmed PEM buffer containing WDL3 or MAP65-1 and incubated for 60 min at 35°C prior to fixation. Samples were fixed with 1% glutaraldehyde for observation by confocal microscopy.

Analysis of WDL3 Promoter: GUS Activity

A DNA fragment of the WDL3 promoter containing 1689 bp upstream of the translation start site was amplified by PCR. The primers used for amplification were 5'-GGATCCCGGGACCGACCATTGA-3' and 5'-GAATTCTTTTCTTGTGACAGACC-3'. The sequence was then cloned into the pCAMBIA1391 vector (Invitrogen). The resulting construct was then transformed into Arabidopsis plants using Agrobacterium tumefaciens (strain GV3101) by the floral dip method (Clough and Bent, 1998). Thirty-seven independent transgenic lines were obtained, and the homozygous seedlings were used for histochemical localization of GUS activity in hypocotyl cells. The GUS staining procedure was performed as previously described by Wang et al. (2007).

Light Treatment

Three-day-old etiolated wild-type, WDL3-overexpressing, or WDL3 RNAi Arabidopsis with a GFP-tubulin background grown on half-strength MS medium were treated with light (33 μmol m-2 s-1) for 30 and 60 min, and cortical microtubules were observed using confocal microscopy.

Generation of WDL3 Overexpression and RNAi Arabidopsis Lines

To prepare stable WDL3 RNAi Arabidopsis lines, a WDL3 RNAi vector was generated by amplifying 306- or 360-bp WDL3 coding sequences in the sense and antisense orientations and inserting them into the pFGC5941 vector. Primers used for amplification of WDL3 RNAi sequences were 5'-ATTAAATCTTCCGATGACTTG-3' and 5'-GGCGCCTCGAAGCTCCTTTTGT-3'; 5'-CGGATCATTTAATCTTCCGATGACTTG-3' and 5'-TCTAGAGGCGGCCCCGCTCAGGTTTTG-3'. The following primers were used for amplification of WDL3 RNAi-1 sequences: 5'-CCATGGATGGAATCTGTGAGAAG-3' and 5'-CTGGATGGAACGACCTTTGCTTGTG-3'; and 5'-GGATCCCATGAGGATATCTGTATGGACAAGG-3' and 5'-TCTAGACTGAGTGAAGCGACTTCTGAGTG-3'.

To stably express WDL3-GFP, the full-length WDL3 cDNA was amplified by PCR and subcloned into the pBI221 vector (Invitrogen), which expressed WDL3 under control of the 3SS promoter and a nopaline synthase terminator. GFP was amplified and inserted at the C terminus of WDL3. The cDNAs encoding WDL3 and GFP were then amplified and subcloned into the pCAMBIA1300 expression vector and transformed into wild-type (Columbia ecotype) Arabidopsis plants with Agrobacterium (strain GV3101). The transgenic homozygous Arabidopsis lines from the T3 generation were used.

RT-PCR and Immunoblot Analysis

RT-PCR and quantitative real-time PCR analysis was performed to assess WDL3 and other WVD2/WDL transcript levels in WDL3 RNAi, RNAi-1, and overexpressing seedlings. Total RNA was isolated using TRIzol reagent (Invitrogen) followed by treatment with RNase-free DNase I (Takara) at 37°C for 30 min to degrade genomic DNA. The treated RNA samples (2 μg each) were used as templates for first-strand cDNA synthesis (Takara). Real-time PCR was performed using Applied Biosystems 7500 real-time PCR system with SYBR Premix Ex Taq (Takara). Relative expression levels were calculated as described (Huang et al., 2010). All experiments were repeated at least three times. Primers were described in Supplemental Table 2 online. The 18S rRNA was also amplified as a loading control using the following primers: 5'-CGGCTACCACTCAGAGGA-3' and 5'-CTGGATGGAACGACCTTTGCTTGTG-3'. Protein extracts were prepared from wild-type and WDL3-GFP transgenic seedlings. Blots were probed with an anti-GFP antibody (Roche) at a dilution of 1:5000 in TBST (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20, pH 7.5) and alkaline phosphatase-conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich) at a dilution of 1:10,000. Actin or nonspecific bands on the blot were used as loading controls.

Measurement of Individual Microtubule Dynamics

To analyze the dynamics of individual microtubules, hypocotyl cells of the wild-type, WDL3-overexpressing, and WDL3 RNAi lines with GFP-tubulin backgrounds were used. Time series images 300 s in length (with 5-s intervals) were obtained under spinning disc confocal microscopy. Measurements were performed using ImageJ tools as described by DeBoit et al. (2007). Microtubules with clearly visible leading plus ends and at least 10 times the number of phase transitions were selected for measurements in the wild-type (n = 36 microtubules from nine seedlings), WDL3-overexpressing (n = 36 microtubules from 20 seedlings), and WDL3 RNAi cells (n = 40 microtubules from 12 seedlings). Rescue and catastrophe event frequencies were measured and analyzed according to Kink et al. (2012). All of the data were processed using Excel software (Microsoft Office 2003).

Ballistics-Mediated Transient Expression in Leaf Epidermal Cells

Subcellular localization of WDL3-GFP and cortical microtubules was visualized using transiently expressed 3SS:WDL3-GFP and 3SS:MBD-mCherry constructs expressed in Arabidopsis (Columbia ecotype) leaf epidermal cells. These experiments were performed as previously...
provided by Fu et al. (2002). We used 1 μg of 35S::WDL3-GFP and 1 μg of 35S::MBD-mCherry DNA for particle bombardment using a PDS-1000/He system (Bio-Rad). Six to 8 h after bombardment, GFP and mCherry signals were detected using a Zeiss LSM 510 META confocal microscope. Filamentous structures containing WDL3-GFP in leaf epidermal cells were visualized after treatment with 10 μM oryzalin for 10 min.

Quantification of Cortical Microtubules
The procedure was performed as previously described by Li et al. (2011). Briefly, we used ImageJ software (http://rsb.info.nih.gov/ij/) to quantify the density of cortical microtubules in the cell. A vertical line was drawn perpendicular to the majority of the cortical microtubules, and the density of cortical microtubules crossing the line was quantified. Measurements were performed in triplicate for each cell, and a minimum of 24 cells per treatment were used. The values were recorded, and significant differences were analyzed using a paired Student’s t test.

Accession Numbers
Sequence data described in this article can be found in the Arabidopsis Genome Initiative under accession numbers At3g23090 (WDL3) and At2g0580 (RPN1a).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. The GFP Tag Does Not Disturb the Physiological Functions of WDL3.

Supplemental Figure 2. Expression of WDL3 Is Associated with the Hypocotyl Phenotype in WDL3 RNAi Lines.

Supplemental Figure 3. Decreased Expression of WDL3 in WDL3 RNAi-1 Arabidopsis Enhances Hypocotyl Growth.

Supplemental Figure 4. Expression of WVD2/WDL Is Unaffected in WDL3 RNAi and RNAi-1 Lines.

Supplemental Figure 5. Etiolated Hypocotyl Length Is Similar in Wild-Type, WDL3 Transgenic, and WDL3 RNAi Seedlings.

Supplemental Figure 6. WDL3 Expression in Arabidopsis Tissues and Organs.

Supplemental Figure 7. Expression and Purification of the GST-Tagged WDL3 Fusion Protein.

Supplemental Figure 8. WDL3 Decorates Cortical Microtubules in WDL3-GFP Transgenic Arabidopsis.

Supplemental Figure 9. Light and Dark Treatments Affect Sensitivity of Cortical Microtubules to Oryzalin.

Supplemental Figure 10. WDL4/5/6 Expression Is Not Regulated by Light at the Protein Level.

Supplemental Table 1. Microtubule Dynamic Parameters in Wild-Type, WDL3-Overexpressing, and WDL3 RNAi Lines.

Supplemental Table 2. Primers Used to Detect Transcripts in the WVD2/WDL Family.

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AUTHOR CONTRIBUTIONS
T.M. designed the project. X.L., T.Q., Q.M., J.S., and Z.L. performed specific experiments and analyzed the data. T.M. wrote the article. M.Y. and T.M. revised and edited the article.

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