

An E3 ubiquitin ligase, ERECT LEAF1, functions in brassinosteroid signaling of rice

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Abbreviations: ARM, ARMADILLO; BL, brassinolide; ELF1, ERECT LEAF1; PUB, plant U-box

A spontaneous rice mutant, *erect leaf1* (*elf1-1*), produced a dwarf phenotype with erect leaves and short grains. Physiological analyses suggested that *elf1-1* is brassinosteroid-insensitive, so we hypothesized that *ELF1* encodes a positive regulator of brassinosteroid signaling. *ELF1*, identified by means of positional cloning, encodes a protein with both a U-box domain and ARMADILLO (ARM) repeats. U-box proteins have been shown to function as E3 ubiquitin ligases; in fact, ELF1 possessed E3 ubiquitin ligase activity in vitro. However, ELF1 itself does not appear to be polyubiquitinated. Mutant phenotypes of 2 more *elf1* alleles indicate that the entire ARM repeats is indispensable for ELF1 activity. These results suggest that ELF1 ubiquitinates target proteins through an interaction mediated by ARM repeats. Similarities in the phenotypes of *elf1* and *d61* mutants (mutants of brassinosteroid receptor gene *OsBRI1*), and in the regulation of *ELF1* and *OsBRI1* expression, imply that ELF1 functions as a positive regulator of brassinosteroid signaling in rice.

Introduction

Brassinosteroids are endogenous phytohormones that are involved in the regulation of various growth and developmental processes in higher plants.¹⁻³ Brassinosteroids have been shown to enhance various aspects of crop production in rice (*Oryza sativa* L.), such as disease resistance and abiotic stress tolerance.^{4,5} Interestingly, overproduction and deficiency of brassinosteroids both increase rice grain yield, although by different mechanisms.⁶⁻⁸ These findings suggest the feasibility of genetic improvement of crop production by the modulation of brassinosteroid function.

Genetic and molecular studies have identified key components of the brassinosteroid signaling pathway, which include membrane receptor kinases (BRI1 and SERKs, including BAK1), intracellular kinases (BIN2 and BSKs) and a phosphatase (BSU1 and PP2A), and nuclear transcription factors (BES1 and BZR1).^{9,10} Subsequent biochemical studies have revealed many details about signaling events from brassinosteroid perception at the cell surface to gene expression in the nucleus.^{9,10} In rice, the putative brassinosteroid receptor gene *OsBRI1* and its loss-of-function mutants (the *d61* mutants) have been identified.¹¹ Although the first 2 *d61* mutants identified were weak alleles, 12 alleles with different severities of phenotype have been identified to date.^{12,13} Two other brassinosteroid-insensitive rice mutants, *dlt* and *lic*, have also been identified.^{14,15} *DLT* encodes a GRAS-family transcription factor and probably acts downstream of a putative rice BZR1 ortholog. *LIC* encodes a CCCH-type zinc

finger protein and probably acts as an antagonistic transcription factor of a rice BZR1 ortholog, *dlt*, *lic-1*, and weak alleles of *d61* all showed semi-dwarf, erect-leaf phenotypes, suggesting that such phenotypes are common in brassinosteroid-insensitive rice mutants.

In this study, we characterized a rice mutant, *erect leaf1* (*elf1-1*), having dwarf and erect-leaf phenotypes. This mutant also had a short-grain phenotype similar to that observed for recently identified *d61* and brassinosteroid-deficient *d2* alleles.^{13,16} *elf1-1* also showed various brassinosteroid-related phenotypes including increased levels of an endogenous bioactive brassinosteroid; therefore, we hypothesized that *elf1* mutants have defects in novel brassinosteroid signaling components. The *ELF1* gene encodes a U-box-containing E3 ubiquitin ligase, and was found to be identical to the recently identified *Taihu Dwarf1* (*TUD1*) gene.¹⁷ Based on these experiments, we concluded that ELF1 functions in brassinosteroid signaling as an ubiquitin E3 ligase.

Results

elf1-1 showed brassinosteroid-insensitive phenotypes

As the result of a large-scale screening of rice mutant collections, we identified several lines that showed the morphological characteristics of brassinosteroid-related mutants, namely, dwarf plant stature and erect leaves. Seven of these mutants were obtained from a Nipponbare library of tissue culture-induced mutations.¹⁸ Three of the 7 mutants were alleles

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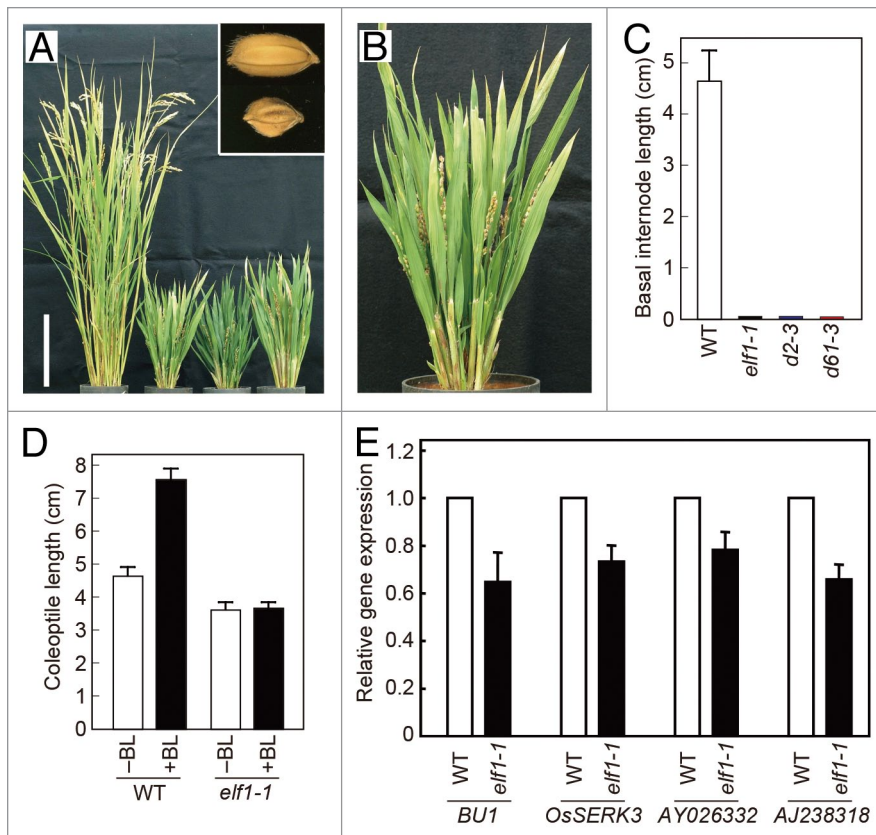


Figure 1. *elf1* mutants showed brassinosteroid-insensitive phenotypes. (A) Comparison of gross morphology between the wild-type (WT) and 3 *elf1* mutants. Left to right: Nipponbare (wild-type), *elf1-1*, *elf1-2*, and *elf1-3*. Bar, 20 cm. Inset represents grain morphology of the wild-type (upper) and *elf1-1* (lower). (B) Close-up view of *elf1-1*. (C) Length of the second lower internode of the wild-type, *elf1-1*, and brassinosteroid-related mutants grown in the dark. Data presented are the means \pm SD of 5 plants. (D) Effect of BL treatment on the coleoptile elongation of the wild-type and *elf1-1*. Data presented are the means \pm SD of 5 plants. (E) Quantitative RT-PCR analysis of brassinosteroid-response genes (*BUI*, *OsSERK3*, *AY026332*, and *AJ238318*). Expression levels were normalized against the values obtained for *Histone H3*. The value obtained from the wild-type was then normalized to a value of 1.0. Data presented are the means \pm SD of 3 biological repeats.

of *d61*,¹³ which is caused by mutations in the brassinosteroid receptor gene *OsBR11*, and another 3 were alleles of *d2*,¹⁶ which is caused by mutations in the brassinosteroid biosynthetic enzyme gene *CYP90D2/D2*. The remaining mutant, *elf1-1*, was not an allele of the previously identified brassinosteroid-related mutants, so we further characterized this mutant.

Like the Nipponbare-derived brassinosteroid-related *d61* and *d2* mutants,^{13,16} *elf1-1* showed a reduction in plant height (to 34% of the wild-type height, $n = 10$, $P < 0.001$) as shown in Figure 1A. In wild-type rice, the leaf blade bends away from the vertical axis of the leaf sheath toward the abaxial side, whereas the leaves of *elf1-1* were completely erect (Fig. 1A and B). The grains of *elf1-1* were visibly shorter and smaller than those of their original strain, Nipponbare (Fig. 1A, inset), similar to the grains of the Nipponbare-derived *d61* and *d2* mutants.^{13,16}

The rice brassinosteroid-insensitive and -deficient mutants show a photomorphogenic phenotype when grown in the dark: the mesocotyl and internodes do not elongate.^{11,19,20} Thus, this unique phenotype is a good criterion with which to determine

whether a novel dwarf mutant is related to brassinosteroids. We grew *elf1-1*, wild-type, brassinosteroid-deficient (*d2-3*), and brassinosteroid-insensitive (*d61-3*) plants in the dark for 2 wk. The basal internodes of the wild-type plants elongated under dark conditions, but elongation did not occur in *elf1-1*, *d2-3*, and *d61-3* (Fig. 1C). The failure of internode elongation in the dark strongly supported the notion that *elf1-1* is a brassinosteroid-related mutant. Next, we measured the effects of brassinolide (BL, a bioactive brassinosteroid) on coleoptile length (Fig. 1D). The coleoptile length of the mutants was not affected by 100 nM BL, whereas the coleoptile length of wild-type plants increased after treatment with 100 nM BL. These results suggested that *elf1-1* plants are less sensitive to exogenous BL than are wild-type plants.

To confirm whether the response to brassinosteroids is suppressed in *elf1-1* plants at the level of gene regulation, we monitored the expression of brassinosteroid-response genes: *BUI*, *OsSERK3*, *AY026332*, and *AJ238318*.^{21–23} The level of *BUI* mRNA in *elf1-1* seedlings was 65% of that in wild-type seedlings (Fig. 1E). Similarly, the expression levels of *OsSERK3*, *AY026332*, and *AJ238318* in the *elf1-1* seedlings were 66–78% of those in the wild-type seedlings. We previously reported that the steady-state level of *BUI* mRNA in Nipponbare-derived mutant of brassinosteroid receptor gene *OsBR11* (*d61-1N*) was about 68% of that in Nipponbare.¹³ Among 12 alleles of *d61* mutants, *d61-1N* showed severely dwarfed stature with erect leaves and short grains as *elf1-1* (plant height of *d61-1N* was reduced to 31% of the wild-type height). Similarities in the gross morphology and the decreased level of *BUI* expression between *elf1-1* and *d61-1N* suggest that *elf1-1* is also a brassinosteroid-insensitive mutant.

Accumulation of bioactive brassinosteroid in *elf1-1*

We also monitored the expression of 6 brassinosteroid-related genes: *OsDWF4*, *D11*, *D2*, *OsCPD1*, *OsDWF*, and *OsBR11*. *OsDWF4* and *D11* encode C-22 hydroxylases (*CYP90B2* and *CYP724B1*, respectively),⁶ *D2* encodes C-23 hydroxylase (*CYP90D2*),²⁴ *OsCPD1* encodes a rice ortholog of C-3 oxidase (*CYP90A3*),^{25,26} *OsDWF* encodes C-6 oxidase (*CYP85A1*),¹⁹ and *OsBR11* encodes the brassinosteroid receptor.¹¹ The expression of these genes is regulated by a homeostatic system that controls bioactive brassinosteroid levels; i.e., expression is increased in brassinosteroid-related mutants and decreased by BL treatment in wild-type.^{6,11,19,20,25} The level of *OsBR11* mRNA in *elf1-1* seedlings was 1.7 times that in wild-type seedlings (Fig. 2A). Similarly, the expression levels of brassinosteroid biosynthetic genes

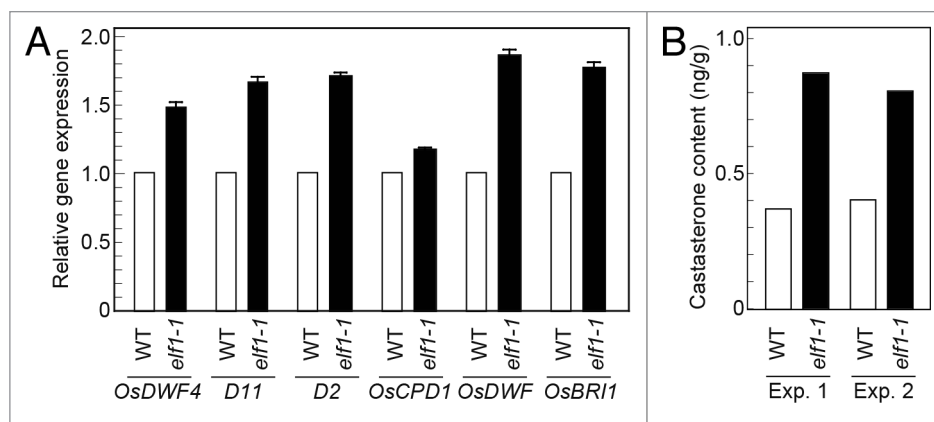


Figure 2. Brassinosteroid biosynthesis was increased in *elf1-1* relative to wild-type (WT). **(A)** Quantitative RT-PCR analysis of brassinosteroid biosynthetic enzyme genes (*OsDWF4*, *D11*, *D2*, *OsCPD1*, and *OsDWF*) and receptor gene *OsBRI1*. Expression levels were normalized against the values obtained for *Histone H3*. The value obtained from the wild-type was then normalized to a value of 1.0. Data presented are the means \pm SD of 3 biological repeats. **(B)** Endogenous content of castasterone in the wild-type and *elf1-1*. Two independent experiments were performed.

(except for *OsCPD1*) in the *elf1-1* seedlings were 1.5–1.8 times those in the wild-type seedlings. The smaller increase in *OsCPD1* expression in the *elf1-1* seedlings (1.2 times that in the wild-type) may be caused by redundancy between the *OsCPD1* and *OsCPD2* genes.²⁵ The level of *CYP85A1* mRNA in *elf1-1* seedlings was 1.8 times that in wild-type seedlings (Fig. 2A). In *d61-1N*, the *CYP85A1* expression was increased to 1.3 times that in the wild-type.¹³ These results indicate that the increased expression of brassinosteroid biosynthetic enzyme genes in *elf1-1* was owing to feed-forward upregulation by the homeostatic system. Consistent with these findings, the endogenous content of castasterone, a bioactive brassinosteroid in rice, doubled in the *elf1-1* seedlings relative to wild-type (Fig. 2B). The accumulation of bioactive brassinosteroid, accompanied by morphological alterations of the types caused by defects in brassinosteroid function, strongly supports our conclusion that *elf1-1* is a brassinosteroid-insensitive mutant and that *ELF1* encodes a positive regulator of brassinosteroid signaling in rice.

The *ELF1* gene encodes a U-box-containing ubiquitin E3 ligase

The *ELF1* locus was cloned using a map-based chromosome walking procedure and mapped to a ~20-kb region on the short arm of chromosome 3 (Fig. 3A). This region includes 2 predicted open reading frames (locus IDs Os03 g0232600 and Os03 g0232800). By comparing the nucleotide sequences of *elf1-1* and the wild-type at each of these loci, we concluded that *ELF1* corresponds to Os03 g0232600, which encodes a protein of 459 amino acid residues in a single exon. There was a 13-bp deletion in the Os03 g0232600 locus of *elf1-1* relative to wild-type, whereas no difference between *elf1-1* and wild-type was detected at the Os03 g0232800 locus. The dwarf and short grain phenotypes of *elf1-1* were complemented by the introduction of a 5.3-kb genomic DNA fragment (from positions –3169 to +2167, taking the translation initiation site as +1) containing the entire candidate gene at the Os03 g0232600 locus (Fig. 3B and C) confirmed that the *elf1-1* phenotype is caused by a loss-of-function mutation in this predicted *ELF1* gene.

Sequence comparison suggested that ELF1 contains a typical U-box domain followed by six ARMADILLO (ARM) repeats (Fig. 3D). This domain organization is frequently found in the plant U-box (PUB) protein family, whose members function as E3 ubiquitin ligases.²⁷ The 13-bp deletion in *elf1-1* produced a frameshift at Asp-58, resulting in a truncated protein of 77 amino acid residues (Fig. 3D). Therefore, *elf1-1* was considered to be a null allele. Based on sequence analysis of *ELF1*, 2 allelic mutants (*elf1-2* and *elf1-3*) were further identified from our mutant collection (Figs. 1A and 3D). *elf1-2* had a single nucleotide substitution (CAG to TAG) that resulted in a substitution of Gln-166 with a stop codon. *elf1-3* had a 5-bp deletion that produced a frameshift at Thr-440, resulting in a truncated protein of 458 amino acid residues. The 3 mutant alleles had similar phenotypes, including severely dwarfed stature with erect leaves and short grains, indicates that mutations of *ELF1* in *elf1-2* and *elf1-3* also caused loss of ELF1 protein function in these alleles.

A BLAST search using the whole amino acid sequence of ELF1 revealed the highest sequence similarity with *Arabidopsis* proteins AtPUB30 and AtPUB31 (63% and 59% identities, respectively). Both AtPUB30 and AtPUB31 were grouped with the class III PUB proteins.²⁷ Phylogenetic analysis of class III PUB proteins from rice, *Arabidopsis*, and potato revealed that class III PUB proteins fall into several clades containing rice and *Arabidopsis* proteins, and ELF1 was distantly related to the other rice class III PUB proteins (Fig. 3E).

ELF1 encodes a functional E3 ubiquitin ligase

Because one of the important features of U-box-containing proteins is to function as E3 ubiquitin ligases, and the U-box is essential for the E3 ubiquitin ligase activity of such proteins,²⁸ we tested whether ELF1 also possessed the characteristics of E3 ubiquitin ligase. First, we examined the interactions between ELF1 and the other components of the ubiquitin protein degradation pathway. To test if an intact U-box domain in ELF1 is required for these interactions, we produced intact ELF1 and U-box domain-deleted ELF1 (deleted from Val-65 to Phe-132; Δ U-box) proteins for yeast 2-hybrid assays (Fig. 4A). The

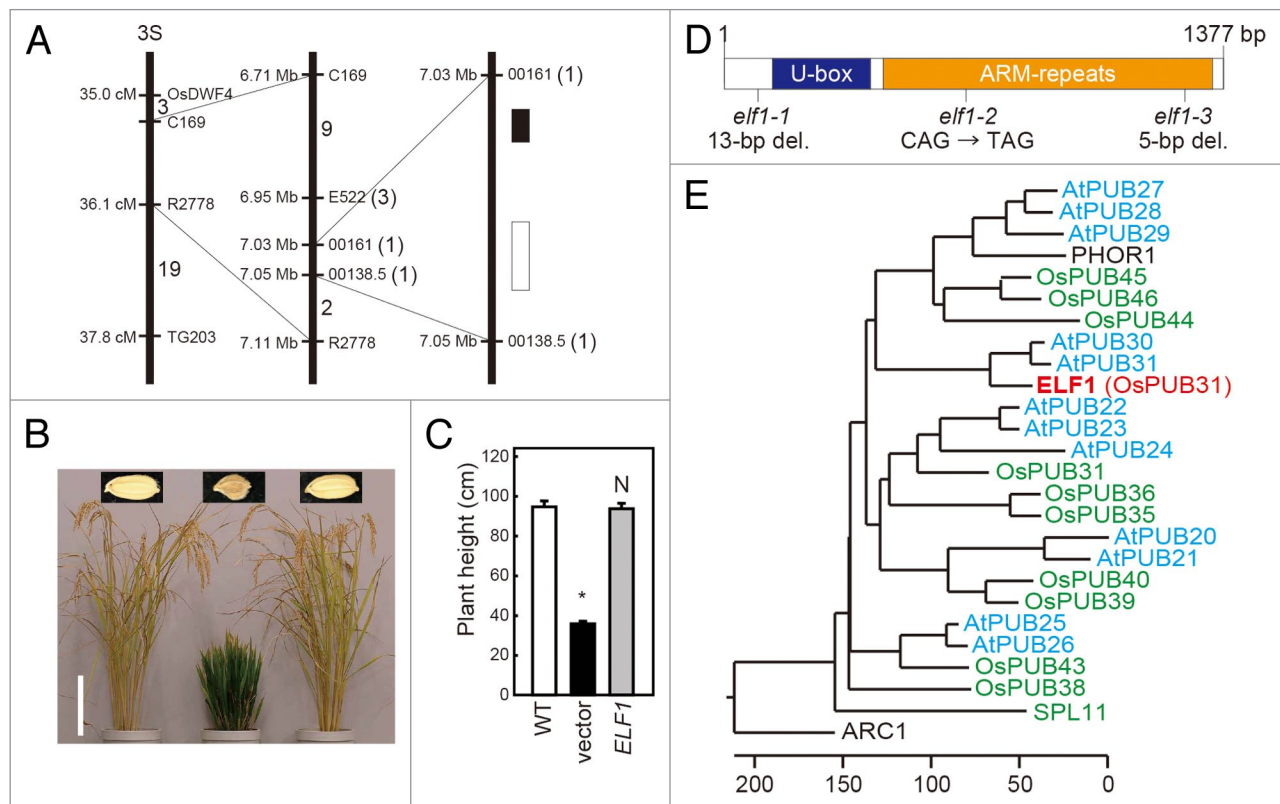


Figure 3. Molecular characterization of *ELF1*. **(A)** High-resolution linkage and physical map of the *ELF1* locus. The horizontal bars represent molecular markers, and the numbers of recombinant plants are indicated on the linkage map. Black and white boxes indicate Os03 g0232600 and Os03 g0232800, respectively. **(B)** Comparison of gross and seed morphologies between wild-type (left) and *elf1-1* mutant transgenic plants containing the empty vector (middle) and a genomic DNA fragment encompassing the entire *ELF1* gene (right). Bar, 20 cm. **(C)** Plant height of wild-type (WT) and *elf1-1* mutant transgenic plants containing the empty vector (vector) and a genomic DNA fragment encompassing the entire *ELF1* gene (*ELF1*). Each bar represents the mean \pm s.d. of 9 different plants (wild-type) and 3 different plants for each of the 3 independent *elf1-1* mutant transgenic lines. Bars labeled with asterisks differ significantly ($P < 0.001$, Dunnett post-hoc test) from wild-type plants. N, not significant. **(D)** Molecular structure of the *ELF1* and positions of the mutations in the 3 *elf1* alleles discovered in the present study. **(E)** Phylogenetic relationships of PUB proteins. OsPUBs, AtPUBs, and PHOR1 are class III PUB proteins from rice, *Arabidopsis*, and potato, respectively. SPL11 and ARC1 are class II PUB proteins from rice and rape, respectively.

intact *ELF1* protein interacted with the ubiquitin-conjugating enzyme OsUBC5b (E2 in Fig. 4B) and polyubiquitin (Poly-Ub in Fig. 4B); however, deletion of the U-box domain in Δ U-box protein greatly reduced these interactions (Fig. 4B).

Next, we wanted to determine whether *ELF1* also possesses E3 ubiquitin ligase activity. We expressed in *Escherichia coli* and affinity-purified the intact *ELF1* protein as a glutathione *S*-transferase (GST) fusion. In the presence of yeast E1 and human E2 Hubs5b, ubiquitination activity was observed for the purified GST-*ELF1* fusion protein (Fig. 4C). No clear protein ubiquitination was detected in the absence of E1, E2, or *ELF1* (Fig. 4C). In vitro ubiquitination analysis also indicated that the E3 ubiquitin ligase activity was completely abolished in the purified GST- Δ U-box fusion protein (Fig. 4C), suggesting that an intact U-box domain is required for *ELF1*'s E3 ubiquitin ligase activity. It was noteworthy that GST-tag-excised *ELF1* (*ELF1* in Figure 4C) did not show auto-ubiquitination, indicating that *ELF1* in the GST-*ELF1* fusion protein ubiquitinated an amino acid somewhere in the GST-tag region. These results indicate

that *ELF1* possesses E3 ubiquitin ligase activity, but *ELF1* itself does not appear to be polyubiquitinated.

Expression of *ELF1* in wild-type and mutant rice

Quantitative reverse-transcription PCR (qRT-PCR) analysis revealed that *ELF1* was preferentially expressed in panicles at flowering time, at moderate levels in vegetative shoot apices, leaf sheaths, leaf blades, and elongating internodes, and at the lowest level in roots (Fig. 5A). Previous observations indicated that the expression of brassinosteroid receptor gene *OsBR1* was downregulated by brassinosteroid application and upregulated in brassinosteroid-insensitive and -deficient mutants.^{14,29} Thus, we examined whether such feedback regulation also occurs with *ELF1*. qRT-PCR analysis revealed that the expression of *ELF1* was slightly decreased by BL treatment (Fig. 5B), whereas it more than doubled in both brassinosteroid-deficient *brd1-2* (a loss-of-function mutant of brassinosteroid biosynthetic enzyme gene *CYP85A1/OsDWARF*)¹⁹ and brassinosteroid-insensitive *d61-3* (a loss-of-function mutant of *OsBR1*),¹² as shown in Figure 5C. Interestingly, *ELF1* expression also increased in dark-grown wild-type rice (Fig. 5C), as previously reported for *OsBR1*.²⁹

the locations of the *elf1-2* and *-3* mutations. Thus, we consider that ELF1 ubiquitinates target proteins through the interaction mediated by its ARM repeats. We plan to identify target proteins of ELF1 in order to elucidate the functions and mechanisms of ELF1-mediated regulation of brassinosteroid signaling in rice.

Materials and Methods

Plant materials, phytohormone treatments, and gene expression analysis

Seeds of wild-type rice (*Oryza sativa* L. cv Nipponbare) and mutants were sown on Murashige and Skoog agar medium and grown in a growth chamber at 28 °C under continuous light or complete darkness for 2 wk. Treatment with 100 nM BL was performed as previously described.⁴⁴ Total RNA extraction, single-strand cDNAs synthesis, and qRT-PCR were performed as previously described.⁴⁴ Expression levels were normalized against the values obtained for *Histone H3*, which was used as the internal reference gene. Primer sequences were listed in a previous report.⁴⁴

Analysis of endogenous brassinosteroid levels

Shoots from wild-type and *elf1-1* mutant plants were harvested 4 wk after germination. Purification and quantification of brassinosteroids were performed according to the method described previously.¹⁶

Molecular cloning, sequence alignment, phylogenetic tree construction, and complementation test

To map *ELF1*, linkage analysis was performed using an F₂ population of ~4000 plants derived from a cross between *elf1-1* (*japonica* variety) and Kasalath (*indica* variety). The nucleotide sequence of the *ELF1* gene (from positions -3648 to +2353, taking the translation initiation site as +1) from Nipponbare and 3 mutant lines was determined by using an ABI377 sequencer (Applied Biosystems) according to the manufacturer's instructions, and analyzed by using the Lasergene software program (DNASar). A BLAST search was performed as previously reported.⁴⁵ Phylogenetic analysis was performed by using the Lasergene software according to the manufacturer's instructions. For complementation of the *elf1-1* mutation, we amplified the wild-type genomic sequence of *ELF1* from -3169 to +2167 (taking the translation initiation site as +1) by PCR and confirmed the sequence of the amplified product. Binary vector construction and rice transformation were performed as previously described.⁴⁴

Yeast 2-hybrid assay

Full-length *ELF1* cDNA and U-box-deleted *ELF1* cDNA were inserted into the pGBKT7 vector (Clontech); OsUBC5b (Os04 g0667800) and polyubiquitin (Os06 g0681400) were inserted into the pGADT7 vector (Clontech). β -galactosidase liquid assays were performed according to the manufacturer's protocol.

Ubiquitin E3 ligase assay

Full-length *ELF1* cDNA and U-box-deleted *ELF1* cDNA were inserted into the pGEX-6P expression vector (GE Healthcare). Recombinant proteins were purified by GST Purification Modules (GE Healthcare). The in vitro ubiquitylation assay was performed by using a Ubiquitylation Kit (Biomol).

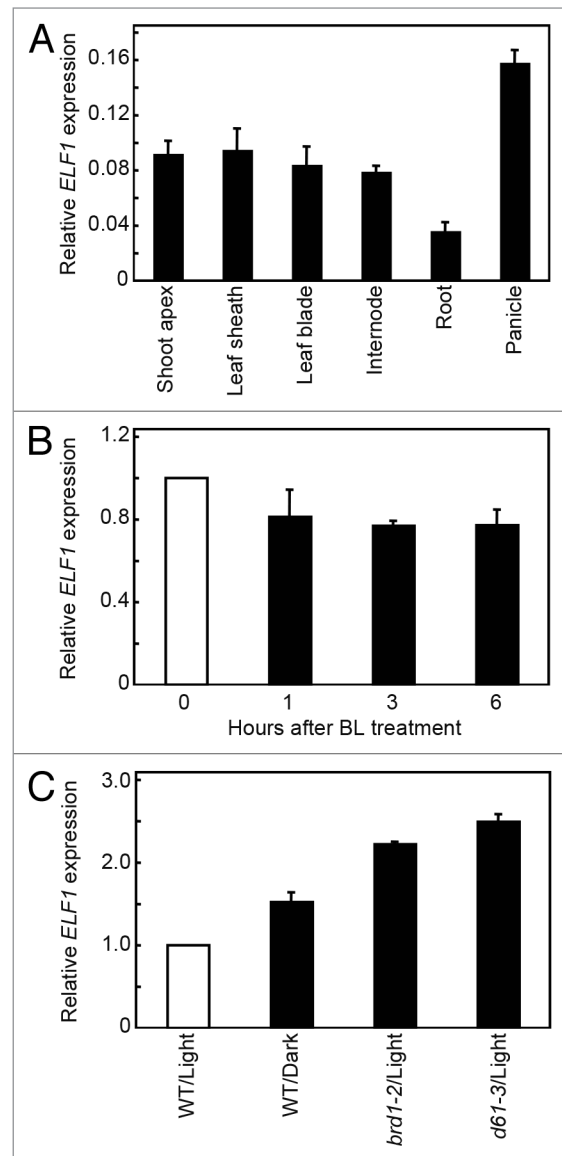


Figure 5. Expression of *ELF1* in wild-type and mutant rice. (A) Relative mRNA levels of *ELF1* in various organs of wild-type rice. (B) Changes in the expression level of *ELF1* in wild-type rice after BL treatment. (C) Wild-type seedlings (WT) were grown under continuous light (Light) or complete darkness (Dark). *brd1-2* and *d61-3* were grown under continuous light. Expression levels were normalized against the values obtained for *Histone H3*. In (B) and (C), the value obtained from the wild-type just before the treatment (B) or grown under continuous light (C) was then normalized to a value of 1.0. Data presented are the means \pm SD of 3 biological repeats.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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