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LETTER TO THE EDITOR

Receptor-like protein ELT1 promotes brassinosteroid signaling through interacting with and suppressing the endocytosis-mediated degradation of receptor BRI1

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Dear Editor,

Phytohormone brassinosteroids (BRs) play important roles in regulating plant development and the components of BR signaling have been widely studied in *Arabidopsis* [1]. Receptor BRI1 (BR insensitive 1, a receptor-like kinase (RLK)) perceives BR signal and is stimulated by BR-induced BRI1-BAK1 (BRI1-associated kinase 1) hetero-dimerization [2, 3] or negatively regulated by BRI1 internalization [4]. Considering that the localization and turnover of BRI1 are independent of BR [5], regulatory mechanism of BRI1 endosomal trafficking is of particular interests. In addition, BR is crucial for regulating rice architecture [6] and there is enormous potential to improve rice yields by manipulating BR level or sensitivity. However, there are only a few components involved in rice BR signaling have been identified and further investigations will help illustrate the mechanism of BR function in monocots and contribute to the molecular breeding of rice architecture.

By phenotypically screening the Shanghai rice T-DNA insertion population [7], enhanced leaf inclination and tiller number 1-D (*elt1-D*, subsequent analysis demonstrated that it is a gain-of-function mutant) displaying reduced height and significantly increased tiller number and leaf inclination (Figure 1A), was identified. Cytological observations (cross-sections or resin sections) showed that exaggerated leaf inclination of *elt1-D* resulted from the increased cell division at adaxial side (Supplementary information, Figure S1A) and decreased sclerenchyma cell layers at abaxial side, of lamina joints (Supplementary information, Figure S1B and S1C), suggesting that ELT1 modulates plant growth possibly by regulating BR signaling.

Heterozygous *elt1-D* plants showed a ~3:1 segregation ratio (*elt1-D* phenotype: wild type) and Southern blot analysis revealed a single T-DNA insertion in the promoter of *Os02g58390* gene (Supplementary information, Figure S2A), which results in an increased expression of *Os02g58390*, especially at lamina joints and tiller buds (Supplementary information, Figure S2B). Suppression of the increased *Os02g58390* expression by RNA interference (Supplementary information, Figure S2C) resulted in the restored growth of *elt1-D* (Supplementary information, Figure S2D-S2H), demonstrating the *Os02g58390/ELT1* effect. Further PCR analysis showed that a knockout mutant containing Tos17 insertion in *ELT1* gene, *elt1* (Supplementary information, Figure S3), is slightly dwarf and exhibits reduced leaf angles and tiller numbers (Figure 1B). Similarly, suppression of *ELT1* expression through RNA interference in wild type caused slightly dwarf plants with erect leaf and fewer tillers (Supplementary information, Figure S4), confirming the essential role of ELT1 in regulating rice growth/architecture.

ELT1 was annotated to encode an inactive RLK with a transmembrane domain and a possible serine/threonine protein kinase domain at C-terminus (Figure 1C). Fluorescence observation of the transiently expressed ELT1-GFP fusion protein in rice protoplasts revealed the ELT1 localization at plasma membrane (Figure 1D). Interestingly, *ELT1* is preferentially expressed at the lamina joint (Figure 1E) and tiller buds (Figure 1F), and transcribed throughout the lamina joint development (Supplementary information, Figure S5A and S5B), which is consistent with the altered leaf inclination and tiller numbers in mutants.

Leaf sheath bending assays showed that *elt1-D* is hypersensitive, while *elt1* and *ELT1-RNAi* are insensitive, to exogenous Brassinolide (BL, Figure 1G), which is confirmed by BR-inhibited root growth assay showing the insensitive or hypersensitive BR response of *elt1* or *elt1-D*, respectively (Supplementary information, Figure S5C). More evidently, western blot analysis showed the increased or reduced ratio of dephosphorylated (active)/phosphorylated (inactive) BZR1 (brassinazole resistant 1) in *elt1-D* or *elt1* (Figure 1H) and reduced expression of BR biosynthesis-related genes in *elt1-D* (Supplementary

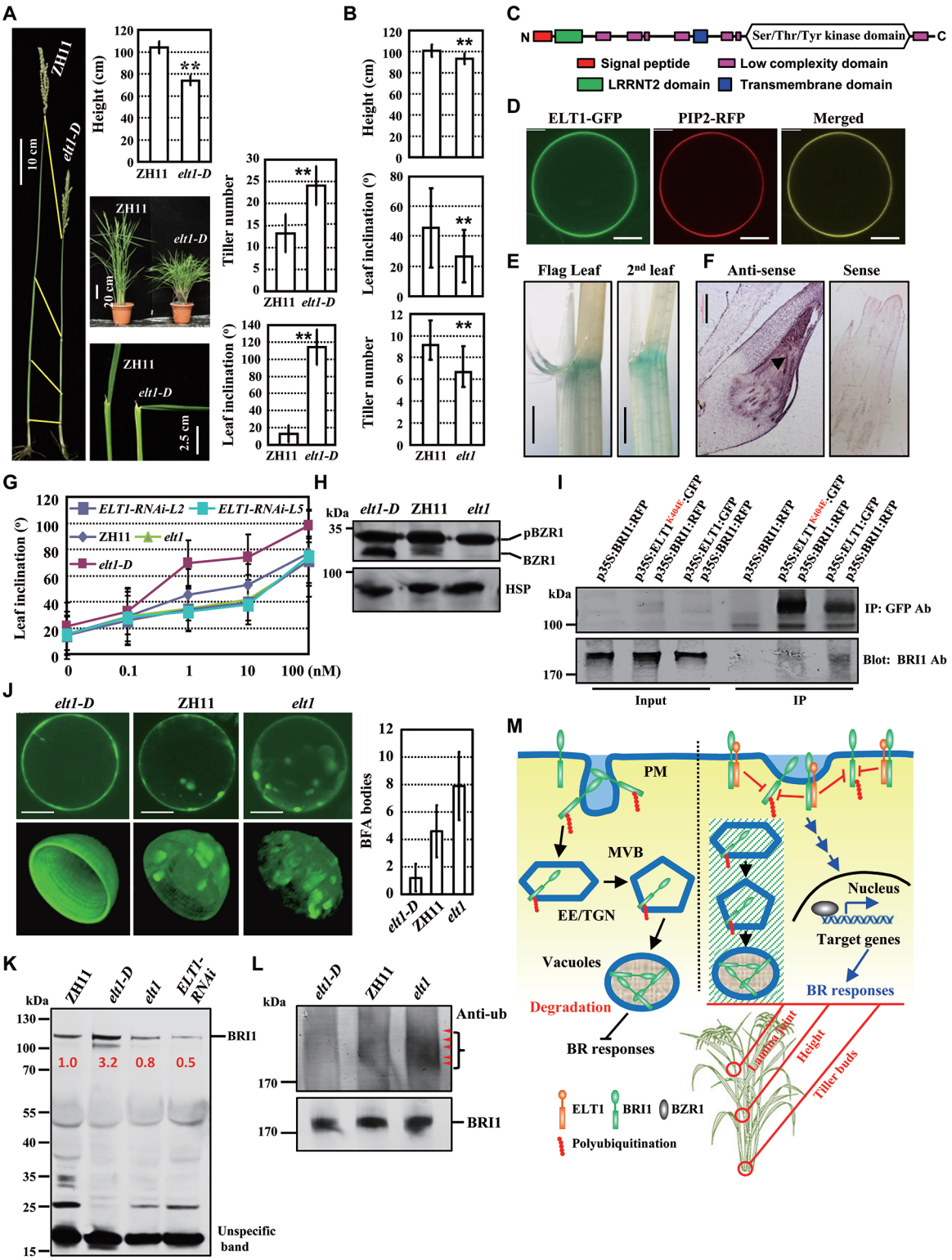
A
ZH11
elt1-D
10 cm
Height (cm)
ZH11 elt1-D
20 cm
Tiller number
Leaf inclination (°)
2.5 cm
B
Height (cm)
Leaf inclination (°)
Tiller number
ZH11 elt1
C
N
Ser/Thr/Tyr kinase domain
C
Signal peptide
Low complexity domain
LRRNT2 domain
Transmembrane domain
D
ELT1-GFP
PIP2-RFP
Merged
E
Flag Leaf
2nd leaf
F
Anti-sense
Sense
G
ELT1-RNAi-L2
ELT1-RNAi-L5
ZH11
elt1
elt1-D
Leaf inclination (°)
0 0.1 1 10 100 (nM)
H
kDa
elt1-D ZH11 elt1
35
pBZR1
BZR1
100
HSP
I
p35S:BRI1:RFP
p35S:ELT1K404E:GFP
p35S:BRI1:RFP
p35S:ELT1:GFP
p35S:BRI1:RFP
p35S:BRI1:RFP
p35S:ELT1K404E:GFP
p35S:BRI1:RFP
p35S:ELT1:GFP
p35S:BRI1:RFP
kDa
100
IP: GFP Ab
170
Blot: BRI1 Ab
Input
IP
J
elt1-D
ZH11
elt1
BFA bodies
elt1-D ZH11 elt1
M
PM
MVB
EE/TGN
Vacuoles
Degradation
BR responses
Nucleus
Target genes
BR responses
Lamina Joint
Height
Tiller buds
ELT1
BRI1
BZR1
Polyubiquitination
K
kDa
ZH11
elt1-D
elt1
ELT1-RNAi
130
100
70
55
40
35
25
15
BRI1
1.0 3.2 0.8 0.5
Unspecific band
L
kDa
elt1-D
ZH11
elt1
170
Anti-ub
170
BRI1

Figure 1 Tissue-specific receptor-like protein ELT1 stimulates BR signaling by suppressing endocytosis-mediated degradation of BRI1 through interaction with BRI1. **(A)** Gain-of-function mutant of ELT1, *elt1-D*, exhibits a dwarf phenotype, increased tiller number and enhanced leaf inclination. ZH11 and *elt1-D* plants at early heading stage were observed. Heights, tiller numbers and degree of leaf inclination of flag leaf were measured and statistically analyzed by student's *t*-test (***P* < 0.01). Data are presented as mean ± SD (*n* > 30) and experiments were repeated three times. **(B)** *ELT1* deficiency results in the reduced height, leaf inclination and tiller numbers. Plant height, tiller number and leaf inclination of flag leaf of ZH11 and knockout mutant *elt1* plants at later heading stage (~15 days after heading) were measured and statistically analyzed by student's *t*-test (***P* < 0.01). Data are presented as mean ± SD (*n* > 30) and experiments were repeated three times. **(C)** Domain structural organization of ELT1. A transmembrane domain, a LRRNT2 domain, and a Ser/Thr/Tyr kinase domain are located at middle, extracellular and intracellular (C-terminal) regions, respectively. **(D)** Fluorescence observation of rice protoplasts expressing ELT1-GFP fusion protein reveals that ELT1 is located at plasma membrane. Plasma membrane aquaporin PIP2-RFP was observed as a positive control. Scale bar, 10 μm. **(E)** Analysis of GUS activity driven by *ELT1* promoter showed that *ELT1* is expressed at the lamina joint of flag leaf and 2nd leaf. Scale bar, 5 mm. **(F)** RNA *in situ* hybridization analysis revealed the *ELT1* transcription at rice tiller buds (highlighted by arrow). Scale bar, 200 μm. **(G)** Compared to ZH11, *elt1-D* or *elt1/ELT1-RNAi* seedlings display increased or reduced lamina joint inclination of etiolated leaves under 24-eBL treatment. Eight-day-old seedlings were analyzed and data are presented as means ± SD (*n* > 50). **(H)** Western blotting analysis revealed the altered amounts of dephosphorylated BZR1 in lamina joints of mature flag leaves of *elt1* or *elt1-D*. Rice HSP was used as internal reference to indicate the equal loading of proteins. **(I)** Co-IP assay confirmed the ELT1-BRI1 interaction *in vivo*. Lysates from transfected rice protoplasts was immunoprecipitated by GFP antibody (upper panel) and detected by BRI1 antibody (bottom panel). After IP, supernatants were analyzed to verify the protein expression (Input, left). Protoplasts transfected with BRI1-RFP was used as a negative control. **(J)** Fluorescence observation revealed the reduced or increased BRI1-containing vesicles of *elt1-D* or *elt1*, compared to those of ZH11 (left, upper). Coleoptile protoplasts of ZH11, *elt1-D* or *elt1* were transfected with BRI1-GFP for 5 h and observed after incubation with CHX (50 μM, 3 h) and BFA (20 μg/ml, 1 h). BFA bodies of BRI1-GFP in *elt1-D*, ZH11 and *elt1* coleoptile protoplasts were quantified (right). Error bars represent SD (*n* = 15). Representative images of z-stack 3D scanning of BRI1-GFP in protoplasts were shown (left, bottom). Scale bar, 10 μm. **(K)** Western blotting analysis reveals the increased or decreased level of BRI1 protein in *elt1-D* or *elt1/ELT1-RNAi* plants. Proteins extracted from ZH11, *elt1-D*, *elt1* and *ELT1-RNAi* flag leaves were analyzed using antibody against BRI1. Band density was measured by Image J and relative quantities were shown. Unspecific band was detected to show the loading of proteins. **(L)** Western blot analysis confirmed the reduced or enhanced ubiquitination of BRI1 in *elt1-D* or *elt1* mutants. Coleoptile protoplasts of ZH11, *elt1-D* or *elt1* were transfected with BRI1-GFP for 12 h and immunoprecipitated using anti-GFP beads. BRI1 in immunoprecipitated fractions and ubiquitination of BRI1 were analyzed using anti-GFP and anti-ub antibody, respectively. **(M)** A schematic model illustrating how ELT1 regulates BR signaling and rice growth. Polyubiquitinated BR receptor BRI1 proteins are degraded through the endocytosis-mediated pathway and tissue-specific ELT1 suppresses the internalization and degradation of BRI1 through interaction with BRI1, resulting in BRI1 accumulation and enhanced BR signaling, and hence the growth regulation of rice height, leaf inclination and tillering. EE/TGN, early endosome/trans-Golgi network; MVB, multivesicular body.

information, Figure S5D), which is consistent with that BR induces BZR1 dephosphorylation [8] and confirms the positive effects of ELT1 on BR signaling.

Considering the similar subcellular localizations of ELT1 and BRI1/BAK1, whether ELT1 stimulates BR signaling by directly interacting with BRI1 or BAK1 is examined. Yeast growth assay showed that ELT1 specifically interacts with rice BRI1 but not BAK1 (Supplementary information, Figure S6A), and by confirming that the ELT1-GFP is functional (Supplementary information, Figure S6B), *in vivo* co-immunoprecipitation (Co-IP) analysis further demonstrated that ELT1 does not interact with BAK1 (Supplementary information, Figure S6C). Bimolecular fluorescence complementation assay (Supplementary information, Figure S6D) and Co-IP analysis (Figure 1I) confirmed the BRI1-ELT1 interaction *in vivo*. Interestingly, the ELT1-BRI1 interaction occurs among intercellular domain (Supplementary information, Figure S6E) but not extracellular domain (Supplementary information, Figure S6F).

Lysine residue of *Arabidopsis* BRI1 (K911) is crucial for BL-dependent increase in association between BAK1 and BRI1. Interestingly, a conserved lysine residue is identified in ELT1 (K404) and mutation of this residue to glutamic acid (K404E) resulted in a significantly decreased ELT1-BRI1 interaction (Figure 1I and Supplementary information, Figure S6E), revealing that ELT1 K404 is important for BRI1-ELT1 interaction.

Although well conserved with enzymatically active serine/threonine kinases, the ELT1 intercellular domain neither exhibits kinase activity (Supplementary information, Figure S7A), nor phosphorylates BRI1 (Supplementary information, Figure S7B) *in vitro*, indicating that ELT1 may not regulate BRI1 through phosphorylation. Considering that transcription level of *BRI1* is not altered in *elt1-D* (Supplementary information, Figure S7C), whether ELT1 regulates BRI1 through internalization was examined by transient expression in protoplasts, which has been demonstrated an appropriate method to study the BRI1 endocytosis [9]. Compared to wild type, much decreased or increased BFA compartments of BRI1-GFP were observed in *elt1-D* or *elt1* protoplasts (Figure 1J), indicating a suppressed BRI1 endocytosis through interacting with ELT1. In addition,

FM4-64 staining confirmed the vesicle-like endosome compartments (Supplementary information, Figure S7D) and general endocytosis in *elt1-D* or *elt1* root cells are not disturbed (Supplementary information, Figure S7E), and yeast three-hybrid and Co-IP analyses revealed that ELT1-BRI1 interaction does not affect the interaction between BRI1 and BAK1 (Supplementary information, Figure S7F and S7G).

Parts of the internalized BRI1 are sorted into late endosomal compartments for vacuolar degradation [4] and altered BRI1 internalization may result in the altered BRI1 levels. Indeed, by using a BRI1-specific antibody (Supplementary information, Figure S8), western blot analysis showed that rice BRI1 protein is significantly accumulated in *elt1-D* and decreased in *elt1* and *ELT1-RNAi* plants (Figure 1K). In addition, as ubiquitination promotes BRI1 internalization, further analysis revealed the attenuated or enhanced BRI1 ubiquitination in *elt1-D* or *elt1* mutant (Figure 1L), indicating that interaction with ELT1 suppresses the BRI1 ubiquitination, leading to the impaired endocytosis-mediated degradation of BRI1 and hence BRI1 accumulation and enhanced BR signaling.

Our study identifies ELT1 as a novel key regulator of rice BR signaling by suppressing BRI1 internalization and suggests a novel function of RLKs lacking kinase activity in regulating the dynamics of membrane proteins, by either subcellular localization or endocytosis. These results will help illustrate the functional mechanism of BRs in monocots, especially in determining distinct agricultural traits of crops (Figure 1M). In addition, the reduced leaf inclination under *ELT1* suppression suggests ELT1 a candidate for rice breeding of designing ideal rice (or other crops) architecture.

Although some components involved in BR signaling have been characterized in rice based on the homologous genes of *Arabidopsis*, there are still functional differences in BR effects. *Arabidopsis* mutants deficient in BR biosynthesis or signaling are dwarf, while *elt1-D*, as well as rice *ili1-D* (ILI1, increased leaf inclination 1) and transgenic rice expressing *pDWF4::Atbes1-D*, exhibits enhanced BR signaling and dwarf plants, suggesting a possible feedback regulation of BR effect in rice height. Indeed, plant-specific RLKs vary largely in terms of structural organization and sequence identity of the extracellular domain [10], and phylogenetic analysis detects two major branches by using ELT1 extracellular domain (Supplementary information, Figure S9; there is no species specificity when using whole ELT1, Supplementary information, Figure S10), further suggesting that ELT1-like clade is probably specific to monocots and BR signaling differs between monocot and dicot plants.

Materials and Methods are available in Supplementary information, Data S1 and Table S1.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)