

Rice *early flowering1*, a CKI, phosphorylates DELLA protein SLR1 to negatively regulate gibberellin signalling

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The plant hormone gibberellin (GA) is crucial for multiple aspects of plant growth and development. To study the relevant regulatory mechanisms, we isolated a rice mutant *earlier flowering1*, *el1*, which is deficient in a casein kinase I that has critical roles in both plants and animals. *el1* had an enhanced GA response, consistent with the suppression of *EL1* expression by exogenous GA₃. Biochemical characterization showed that *EL1* specifically phosphorylates the rice DELLA protein SLR1, proving a direct evidence for SLR1 phosphorylation. Overexpression of SLR1 in wild-type plants caused a severe dwarf phenotype, which was significantly suppressed by *EL1* deficiency, indicating the negative effect of SLR1 on GA signalling requires the *EL1* function. Further studies showed that the phosphorylation of SLR1 is important for maintaining its activity and stability, and mutation of the candidate phosphorylation site of SLR1 results in the altered GA signalling. This study shows *EL1* a novel and key regulator of the GA response and provided important clues on casein kinase I activities in GA signalling and plant development.

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Introduction

The switch from vegetative to reproductive growth is a critical event in the life cycle of flowering plants and is essential for their maximum reproductive success (Bernier, 1988). In *Arabidopsis*, a model plant for eudicots, four major pathways are involved in the control of flowering time: the

photoperiod, autonomous, vernalization, and gibberellin (GA) pathways. In contrast to *Arabidopsis*, rice is a short-day plant. Several rice genes in the photoperiod pathway controlling heading date (*Hd*) (flowering time) have been genetically characterized, including *Se1* (photoperiod sensitivity 1; Yokoo *et al.*, 1980; Yamagata *et al.*, 1986), *Se3–Se7* (Yamagata *et al.*, 1986; Poonyarit *et al.*, 1989; Sano, 1992; Yokoo and Okuno, 1993), and *E1–E3* (*Hd* 1–3, Tsai, 1995; Kinoshita, 1998). Some rice genes encode proteins similar to those in the *Arabidopsis* long-day pathway. Specifically, the products of *Hd* 1, 3a, and 6 are homologous to *Arabidopsis* CO (Yano *et al.*, 2000), FT (FLOWERING LOCUS T, Kojima *et al.*, 2002), and a subunit of kinase CK2 (a regulator of circadian rhythm and flowering time, Takahashi *et al.*, 2001), respectively. In addition, rice phytochromes are also involved in the regulation of flowering time in response to day length (Izawa *et al.*, 2000). Similar to *Arabidopsis*, circadian-regulated *OsGI* expression in transgenic rice has striking effects on flowering time (Hayama *et al.*, 2003).

Arabidopsis mutants defective in GA biosynthesis or signalling show severe delayed flowering (Moon *et al.*, 2003; Yu *et al.*, 2004); however, the role of GA in rice flowering is less clear. Biochemical and genetic studies have characterized key components, especially DELLA proteins, in the GA signalling cascade in both *Arabidopsis* (Peng *et al.*, 1997; Dill and Sun, 2001; Lee *et al.*, 2002; Hussain *et al.*, 2005) and rice (Ikeda *et al.*, 2001). The *Arabidopsis* DELLA proteins GAI, RGA, and RGL1 negatively regulate flowering time in the absence of GA (Mouradov *et al.*, 2002). Recently, the GA receptor *GID1* was identified in *Arabidopsis* (Griffiths *et al.*, 2006; Nakajima *et al.*, 2006) and rice (Ueguchi-Tanaka *et al.*, 2005), and found to interact with DELLA proteins (GAI, RGA, or SLR1) in a GA-dependent manner both *in vitro* and *in vivo* (Willige *et al.*, 2007; Ueguchi-Tanaka *et al.*, 2007a,b). The GA–*GID1*–SLR1 complex is targeted for ubiquitination by SCF^{GID2}, an F-box protein, and degraded by the ubiquitin-dependent proteasome pathway (Sasaki *et al.*, 2003; Gomi *et al.*, 2004; Ueguchi-Tanaka *et al.*, 2005, 2007a,b), which in turn results in the activated GA response.

Casein kinase I, a serine/threonine protein kinase, is a multifunctional protein kinase detected in most eukaryotic cells (Gross and Anderson, 1998). In mammalian cells, there are five isoforms of casein kinase I: α , β , γ , δ , and ϵ (Fish *et al.*, 1995). They are involved in multiple signalling pathways, including vesicular trafficking (Panek *et al.*, 1997; Murakami *et al.*, 1999), growth and morphogenesis (Robinson *et al.*, 1993), circadian rhythm (Kloss *et al.*, 1998; Peters *et al.*, 1999), DNA-repair (Dhillon and Hoekstra, 1994), and cell cycle progression, and cytokinesis (Behrend *et al.*, 2000). Previous studies showed that CKI regulates BR signalling

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in rice (Liu *et al.*, 2003) and cell-to-cell communication (Lee *et al.*, 2005), as well as modifying cell shape by phosphorylating tubulins in *Arabidopsis* (Ben-Nissan *et al.*, 2008, 2010; Lee, 2009). Although a previous report showed that Hd6 (CK2) participates in rice Hd control, there has been no further study on how casein kinase is involved in the regulation of rice flowering time.

From a rice mutant population that we have generated (Fu *et al.*, 2009), an early flowering mutant, *earlier flowering1* (*el1*), was identified. Functional characterization revealed that *EL1*, which encodes a casein kinase I, could phosphorylate the rice DELLA protein SLR1 *in vitro* and *in vivo*, stabilize SLR1, and sustain its activity *in vivo*, to negatively regulate the GA signalling.

Results

Identification of the rice mutant *early flowering1* (*el1*)

To understand the molecular mechanisms underlying heading, the rice mutant *early flowering1* (*el1*) was identified from the Shanghai rice T-DNA insertion population (<http://ship.plantsignal.cn>, Fu *et al.*, 2009). Under normal growth conditions (12 h light/12 h dark cycle), *el1* flowered 5–6 days earlier than the wild type (WT), with a much slower leaf emergence rate (Figure 1A; Supplementary Figure 1A). At various time points, the *el1* plants showed stimulated development of young panicles and the formation of inflorescence meristems (Figure 1B), which is consistent with the analysis on the transcription of floral organ identity genes that

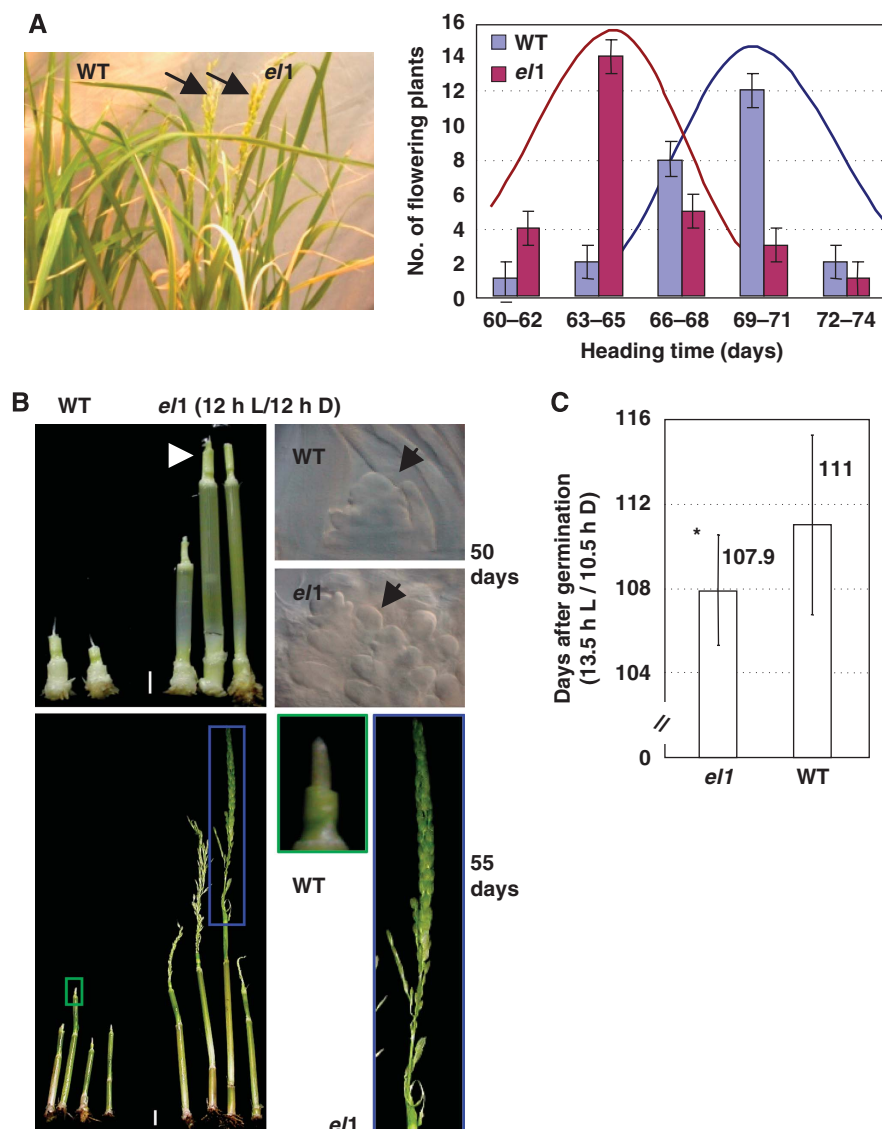


Figure 1 Phenotypic analysis of rice *early flowering 1* (*el1*). (A) Phenotypic observation (left) and analysis of the frequency distribution of heading date (right) indicated the earlier flowering of *el1* plants. The plants were grown in a greenhouse with a 12 h light (L)/12 h dark (D) period. (B) Observations of the inflorescence development of WT and *el1* plants at 50 or 55 days confirmed the earlier flowering of *el1* (Bar = 5 mm). The internodes of *el1* were more elongated and the young panicles in the main culms were more obvious at 50 days of cultivation compared with that of WT. After 55 days of cultivation, the young panicles of *el1* plants were almost fully developed—much earlier than WT. The floral meristems were observed through DIC or highlighted (squared). (C) Analysis of the heading date of *el1* and WT plants under long-day treatment (13.5 h L/10.5 h D) showed that flowering of *el1* is not modulated by photoperiod. The heading time was calculated and statistically analysed using a heteroscedastic *t*-test ($*P < 0.05$, $n = 10$).

revealed advanced or enhanced expression of *Hd6*, *Hd1*, and *OsMADS1* at 40, 45, 50, and 55 days of cultivation in *el1* (Supplementary Figure 1B). This further confirmed the earlier floral initiation of *el1* plants. Interestingly, although most rice early flowering mutants are regulated by photoperiod, measurement of flowering time under long-day treatment (13.5 h light/10.5 h dark) showed similar responses of *el1* and WT plants (Figure 1C), indicating that the function of *EL1* is independent of the photoperiod.

EL1 encodes a casein kinase I

Segregation analysis ($\chi^2_{[3:1]} = 3.44$, $0.1 < P < 0.5$) indicated that *el1* was a recessive mutation. Further analysis of the DNA sequence flanking the T-DNA insertion, obtained by thermal asymmetric interlaced (TAIL)-PCR analysis, revealed a T-DNA insertion in the 15th exon of rice gene Os03g57940, which is designated EL1 and encodes a putative casein kinase I (a 707-amino acid polypeptide, Mw 79.8 kDa, Figure 2A).

Homologous analysis showed that EL1 shows high similarity to other casein kinase I members. Analysis of the phylogenetic relationship between *EL1* and other CKIs indicated that EL1 is closest to yeast YCK2 and CKI (Supplementary Figure 2A, upper panel). Structural organization analysis of EL1 revealed the presence of a nuclear localization signal (NLS), a highly conserved S/T kinase domain, and conserved motifs in CKI (Figure 2A, lower panel; Supplementary Figure 2B) that is identical to that previously identified rice CKI1 (Liu *et al.*, 2003). Biochemical studies of enzymatic activity showed that recombinant EL1 from *Escherichia coli* was able to phosphorylate the partially dephosphorylated casein but not for BSA *in vitro* (Figure 2B), indicating that EL1 is likely an active CKI.

Expression pattern analysis by quantitative real-time RT-PCR (qRT-PCR) showed that *EL1* was transcribed in various tissues, including root, stem, leaf, seedling, and ripened seeds (Figure 2C). Although *EL1* was expressed at relatively low levels in flowers, it was specifically transcribed at stage 2 during floral development (Supplementary Figure 3).

Transient expression of an EL1-GFP fusion protein in onion epidermal cells revealed that EL1 protein is accumulated in the nucleus (Figure 2D), consistent with the presence of an NLS sequence in EL1.

T-DNA insertion resulted in the defective expression of *EL1* (Figure 2E) and expression of the full-length *EL1* cDNA driven by its own native promoter in *el1* (Figure 2E) rescued the early flowering phenotype (Figure 2F) showing the negative effects of *EL1* in controlling rice flowering time.

EL1 is regulated by GA and *el1* shows an increased response to GA

As GA is involved in controlling flowering time in *Arabidopsis*, we tested whether *EL1* expression was regulated by hormones. The results showed that *EL1* expression was evidently and rapidly suppressed by GA treatment (100 μ M GA₃) and declined to a minimal level after 3 h treatment (Figure 3A), suggesting that enzymatic activity of EL1 is regulated in a GA-dependent manner.

GA stimulates plant height and indeed *el1* plants were consistently taller than WT, especially the uppermost internode (UI) (Figure 3B). Further measurement of cell lengths and widths under uniconazole treatment showed that, compared with WT, the elongated longitudinal lengths of the

epidermal cells (at the middle section of the second leaf sheath) were less suppressed (Table I). Leaf sheath growth exhibited an enhanced response to GA₃ (Figure 3C, left panel; Supplementary Figure 5A) and a suppressed response to uniconazole (Figure 3C, middle panel; Supplementary Figure 5B), whereas the transgenic WT plants overexpressing *EL1* consistently resulted in suppressed responses to GA₃ (Figure 3C, right panel; Supplementary Figures 4 and 5C), further supporting the negative effects of EL1 on GA response. Being consistent, calculation of the relative length of the second leaf sheaths of 7-day-old seedlings showed that complementary expression of EL1 in *el1* rescued the enhanced GA response of *el1* (Figure 3D; Supplementary Figure 5D).

This is further demonstrated by the observation that *el1* seeds had much higher α -amylase activity in the absence of GA₃ and that the inhibitory effects of ABA were significantly suppressed (Figure 3E; Supplementary Figure 6), consistent with the altered transcription of rice GA-biosynthesis-related genes *GA20ox2* (suppressed expression) and *GA2ox3* (enhanced expression, Figure 3F). These results confirm the enhanced responses of *el1* to GA and hence the negative role of EL1 in GA signalling.

EL1 phosphorylates the rice DELLA protein SLR1

Previous studies have shown that CKI could regulate the relevant signalling pathway by phosphorylating the downstream proteins; we thus searched for candidate substrates of *EL1* in GA signalling with the help of a computational prediction program (http://scansite.mit.edu/motifscan_seq.phtml). The rice DELLA protein SLR1, which has been shown as a key regulator in GA signalling, was identified. Computational analysis indicated the predicted phosphorylation sites at Ser196 and Ser510 (Supplementary Figure 7). Indeed, yeast two-hybrid analysis revealed that EL1 and SLR1 could interact with each other (Figure 4A), and an in-gel kinase assay indicated that SLR1 can be phosphorylated by EL1 *in vitro* in an EL1-specific manner (Figure 4B). Interestingly, either the N- or the C-termini of SLR1 can be phosphorylated by EL1 (Figure 4B), which is consistent with the presence of the predicted phosphorylation sites at Ser196 and Ser510 and suggests that EL1 may differentially regulate SLR1 by phosphorylating its N- or C-termini.

EL1 phosphorylation of SLR1 is critical for sustaining its activity and stability

Further *in vivo* analysis using transgenic WT or *el1* plants overexpressing SLR1 (Supplementary Figure 8A) indicates that although the protein level of SLR1-YFP fusion protein was almost same (Figure 5A), the effects of SLR1 were significantly suppressed under *EL1* deficiency. Analysis of the lengths of seedlings, mature plants, and second leaf sheaths showed that SLR1 overexpression resulted in a severe dwarf phenotype and shortened second leaf sheaths in WT plants; these effects were suppressed in *el1* plants (Figure 5B; Supplementary Figure 8B). This trend was more significant in mature plants (Figure 5B, right panel; Supplementary Figure 8B, bottom panel), indicating that phosphorylation of SLR1 by EL1 is critical for its function. In addition, analyses of the expression levels of *GA20ox2* and *GA2ox3* showed that *GA20ox2* expression was increased and *GA2ox3* expression was decreased when SLR1 was overexpressed in WT plants,

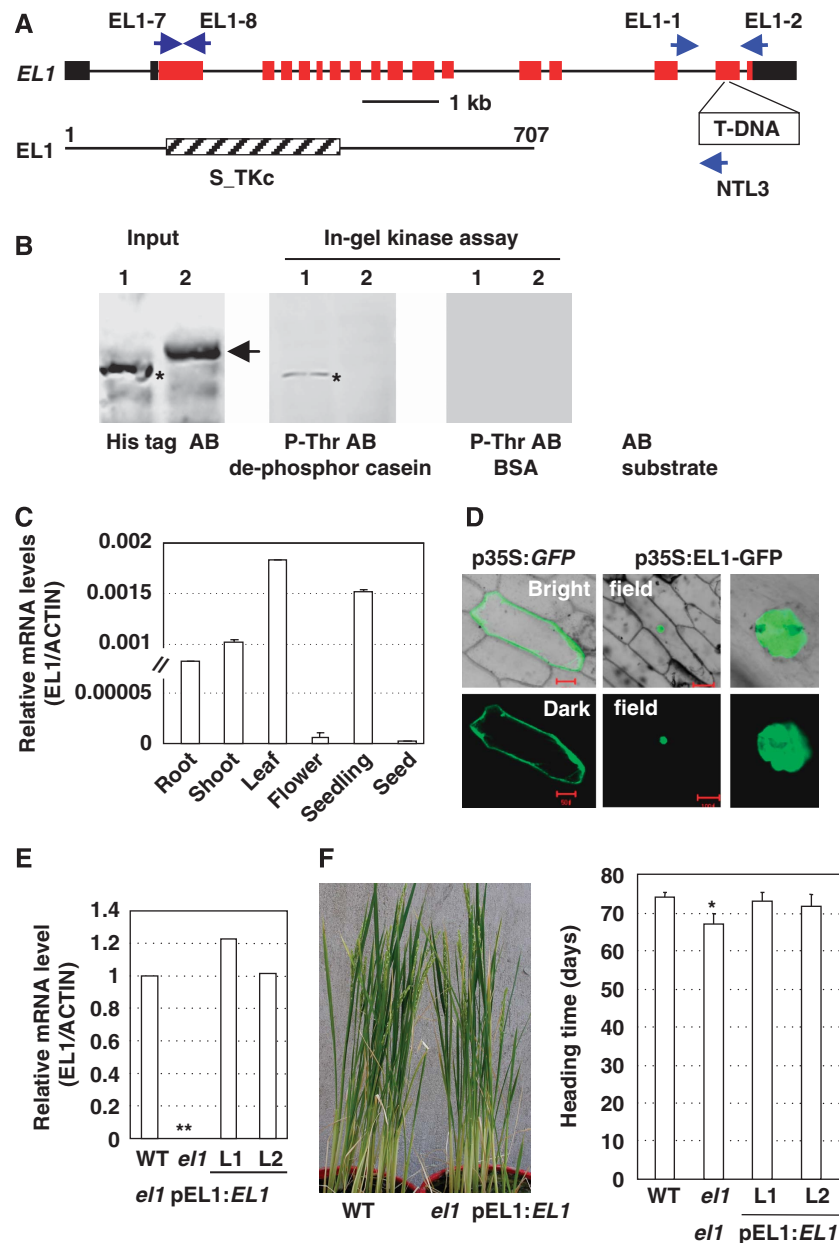


Figure 2 EL1 encodes a casein kinase I and is regulated by GA. (A) Scheme of the *EL1* gene. Exons (red boxes), introns (lines), and UTR (untranslated region, black boxes) are indicated. The T-DNA is inserted at the 15th exon of *EL1*. Positions of primers used for confirming the T-DNA insertion (EL1-1 and NTL3), for identifying the homozygous mutant lines (EL1-1 and EL1-2), and examining the transcripts of EL1 by qRT-PCR analysis (EL1-7 and EL1-8) are indicated. The Thr_Ser protein kinase domain is located in the middle region (lower panel). (B) In-gel kinase assay revealed that recombinant expressed EL1 shows the casein kinase I activity, which could specifically phosphorylate the dephosphorylated casein (middle, asterisk) *in vitro*. Protein of EL1 (lane 1, asterisk) or AtPIP5K9 (lane 2, arrowhead, a kinase control) was used for the assay. Dephosphorylated casein or BSA was used as substrate. The input of EL1 and AtPIP5K9 was detected by His tag antibody (left) and the kinase assay was detected by Thr-P antibody (AB). (C) qRT-PCR analysis revealed the expression of *EL1* in roots, shoots, leaves, and seedlings and relatively lower expression in flowers and seeds. The rice *ACTIN* gene was amplified and used as an internal positive control. (D) Transient expression of EL1-GFP fusion protein in onion epidermis cells through particle bombardment revealed that EL1 is localized in the nuclei. Bar = 20 μ m (left) or 50 μ m (middle). (E) qRT-PCR analysis on the *EL1* expression in homozygous *el1* plants and *el1* plants transformed with *EL1* under its own promoter (pEL1:EL1) revealed the deficient or complemented expression of *EL1* in *el1* plants or *el1* plants transformed with pEL1:EL1. The rice *ACTIN* gene was amplified and used as an internal positive control. (F) Phenotypic observation (left) and calculation (right) revealed that complementary expression of *EL1* recovered the earlier flowering time of *el1* plants (compared with WT), confirming the role of *EL1* in controlling the heading time. The heading time was calculated and statistically analysed using a heteroscedastic *t*-test ($*P < 0.05$, $n = 10$).

whereas the opposite expressions were detected in the *el1* mutant (Figure 5C) showing the effects of EL1 phosphorylation on SLR1 activity. In addition, the *el1* plants overexpressing *SLR1* (*el1* p35S:SLR1-YFP) were much taller than WT plants overexpressing *SLR1* (WT p35S:SLR1-YFP), correlat-

ing with the reduced *GA20ox2* and increased *GA2ox3* expressions in these lines, which suggests that EL1 may take part in the feedback regulation of these genes.

Aside from the effects of phosphorylation by EL1 on SLR1 activity, possible effects of EL1 on SLR1 protein localization

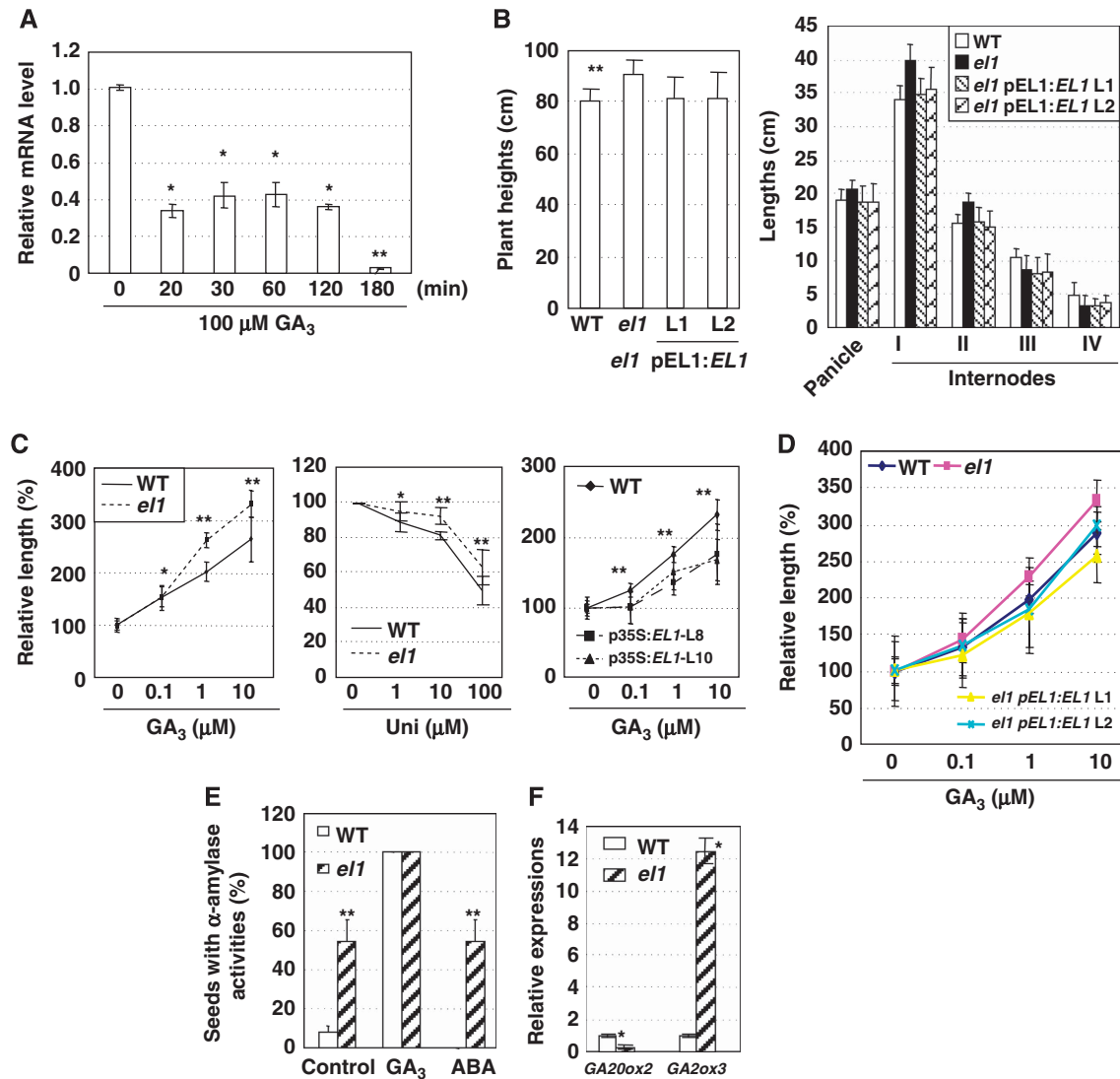


Figure 3 *el1* shows increased response to GA. (A) qRT-PCR analysis revealed that the expression of *EL1* is rapidly suppressed by exogenous GA₃ (100 μM) treatment for 20, 30, 60, 120, or 180 min. The *EL1* expression without GA₃ treatment is set as 1.0. The rice *ACTIN* gene was amplified and used as an internal positive control. Seven-day-old seedlings were used for treatment and analysis. (B) Mature *el1* plants are higher than WT (whereas complemented expression of *EL1* rescued the phenotype, left panel) and have more elongated uppermost internodes (right panel). The lengths of internodes and panicles are calculated and statistically analysed using the heteroscedastic *t*-test (compared with WT, **P* < 0.05; ***P* < 0.01, *n* = 6–10). Data are presented as mean ± s.e. (C) Relative length of the second leaf sheaths of 7-day-old *el1* seedlings in the presence of exogenous GA₃ (0, 0.1, 1, or 10 μM, left) or uniconazole (0, 1, 10, 100 μM, middle), or that of transgenic plants overexpressing *EL1* (p35S:*EL1*) in the presence of exogenous GA₃ (right). The lengths of the second leaf sheaths were measured and the relative length was calculated and statistically analysed using a heteroscedastic *t*-test (**P* < 0.05; ***P* < 0.01, *n* = 15–20). (D) Calculation of the relative length of the second leaf sheaths of 7-day-old WT, *el1* plants, and *el1* with complementary expression of *EL1* (pEL1:*EL1*, lines L1 and L2) in the presence of exogenous GA₃ (0, 0.1, 1, or 10 μM). Error bars represent s.d. (*n* = 15–20). (E) Analysis of α-amylase activities using a starch-containing plate in the presence of GA₃ (1 mM) or ABA (10 μM) for 2 days. The number of seeds secreting α-amylase was calculated and statistically analysed using a heteroscedastic *t*-test (***P* < 0.01, *n* = 20–30). (F) qRT-PCR analysis revealed the suppressed expression of *GA20ox2* and enhanced expression of *GA20ox3* in *el1*.

and stability were studied. Compared with the nuclear localization in WT, reduced phosphorylation of SLR1 in *el1* did not alter its sub-cellular localization (Figure 5D). However, detailed studies showed that GA-mediated SLR1 degradation was greatly enhanced in *el1*. Fluorescence observation and western blot analysis revealed that in comparison with WT the degradation of SLR1-YFP fusion protein after GA₃ treatment was significantly enhanced in *el1* (Figure 5E). This indicates that phosphorylation of SLR1 by EL1 is crucial for its stability.

Pervious studies showed that the SLR1 N-terminal is the regulatory domain and C-terminal is responsible for the repression activity (Itoh *et al*, 2002). To further confirm the effects of phosphorylation on SLR1, point mutation of Ser196 and Ser510 into either A (short for Ala, which results in the defective phosphorylation on this site) or D (short for Asp, which results in the constitutive phosphorylation on this site) was performed and GA signalling of plants containing the corresponding mutated SLR1 was examined by detecting the transcripts of GA synthesis genes *GA20ox2* and *GA3ox2*. The

Table I Length of the epidermal cells in the second leaf sheath of 1-week-old *el1* and WT plants under uniconazole treatment

Length (μm)	Uniconazol (μM)			
	0	1	10	100
WT	111.23 \pm 27.99	93.84 \pm 26.58	86.69 \pm 28.73	39.32 \pm 31.81
<i>el1</i>	105.45 \pm 21.69	109.54 \pm 30.59*	101.21 \pm 37.26**	99.46 \pm 27.47**
<i>Width</i> (μm)				
WT	12.54 \pm 2.42	8.63 \pm 1.52	11.09 \pm 1.89	10.80 \pm 1.88
<i>el1</i>	11.57 \pm 2.16	9.60 \pm 2.00	10.59 \pm 1.83	12.87 \pm 1.93
<i>Length/width</i>				
WT	8.87 \pm 2.79	8.16 \pm 3.54	7.82 \pm 2.91	3.64 \pm 2.09
<i>el1</i>	9.11 \pm 3.86	9.99 \pm 3.09*	9.56 \pm 2.54**	7.73 \pm 2.48**

Data are presented as mean \pm s.e. ($n = 10$). Statistical analysis indicated significant differences (* $P < 0.05$; ** $P < 0.01$).

results showed that after GA₃ treatment, suppressed phosphorylation of SLR1 at either Ser196, Ser510, or both Ser196/Ser510 indeed resulted in the significantly enhanced GA signalling (evidenced suppressed expression of *GA20ox2* and *GA3ox2*), whereas the constitutive phosphorylation of SLR1 at Ser510 or both Ser196/Ser510 would result in the suppressed GA signalling (enhanced expression of *GA20ox2* and *GA3ox2*, Figure 6A). These further confirmed the importance of phosphorylation of SLR1, especially at sites Ser196 or Ser510, on the SLR1 effects in GA signalling. The constitutive phosphorylation of SLR1 at Ser196 did not affect the GA signalling, which might be due to the N-terminal of SLR1, which is mainly for the regulatory domain (Itoh *et al.*, 2002).

Discussion

EL1, a casein kinase, is a novel regulator of GA signalling and has important functions in controlling rice flowering time by regulating GA responses

As an important process in plant reproduction, flowering time is finely controlled by a complex network. In *Arabidopsis*, the key regulators, FT and SOC1, regulate the expression of floral identity genes including *AP1*, *LFY*, and *AG* (Mouradov *et al.*, 2002); most of these genes are *MADS* box genes and determine the identity of floral organs (Yanofsky *et al.*, 1990; Weigel *et al.*, 1992; Jofuku *et al.*, 1994). In rice, the major floral initiation pathway is the photoperiod pathway; five QTLs (Hd1, Hd2, Hd3, Hd5, and Hd6) were found to confer photoperiod sensitivity (Lin *et al.*, 2000; Takahashi *et al.*, 2001). Genetic analysis of the relationship between *EL1* and *Hd* genes showed that *hd1*, *hd3a*, or *hd6* plants with suppressed *EL1* expressions still flower early, similar to *el1* (Supplementary Figure 9). This finding indicates that *EL1* is epistatic to the *Hd* genes, consistent with the phenotypic observation that *el1* has similar photoperiod responses to WT.

GA is crucial for rice flower development and deficiency of GA results in severe dwarfism and failed grain setting in rice (Sakamoto *et al.*, 2003). Although GA is critical in flowering control in the *Arabidopsis*, there is no report of its role in rice flowering. Deletion of a DELLA domain (such as the *gai* allele) will cause delayed flowering even in the presence of GA in *Arabidopsis* (Peng *et al.*, 1997); however, no difference was detected in the flowering time of rice. In the *GAMYB*-deficient mutant, despite shortened internodes and defects in floral organ development, a notable defect in pollen

development was observed (Kaneko *et al.*, 2004). There was no change in the heading date in the *slr1* mutant (Itoh *et al.*, 2002) or *Slr1-d3* (a GA-insensitive mutant, Chhun *et al.*, 2007). Recent studies revealed that GA-deficient plants exhibit shortened UI and panicles enclosed by flag leaves (Yin *et al.*, 2007), suggesting that GA may influence rice heading time by modulating UI elongation. This study provides direct evidence that GA signalling regulates rice flowering and identifies *EL1* as a novel regulator of flowering time by affecting GA responses.

DELLA proteins are important in GA signal transduction (Peng *et al.*, 1997). Truncating the DELLA domain of rice SLR1 suppresses SLR1 degradation under GA treatment (Itoh *et al.*, 2002). Recent studies showed that suppression of SLR1 activity could be accomplished by GA and *GID1* alone, without the F-box protein *GID2* (Ueguchi-Tanaka *et al.*, 2008). These results suggest that other unknown factors might interact with SLR1 to induce its suppressive activity (Ueguchi-Tanaka *et al.*, 2008). This study is the first direct report showing that SLR1 can be phosphorylated by casein kinase I and that *EL1* modulates GA signalling by phosphorylating the rice DELLA protein SLR1.

Phosphorylation of SLR1 is important for the regulation of its activity and stability

Although DELLA proteins have inhibitory roles in the GA response (Itoh *et al.*, 2005), it remains unclear how phosphorylation of DELLA proteins affects their function and/or stability, although this question has been addressed by several research groups (Itoh *et al.*, 2002, 2005; Ueguchi-Tanaka *et al.*, 2008).

It was reported that in the GA signalling pathway, phosphorylation may inactivate DELLA function (Itoh *et al.*, 2002). Furthermore, some kinases can phosphorylate SLR1 within the polyS/T/V, DELLA, and TVHYNP domains (Itoh *et al.*, 2005). However, phosphorylation of SLR1 is independent of its degradation and is not necessary for the interaction of SLR1 with the *GID2*/F-box protein (Itoh *et al.*, 2005). The relationship between SLR1 degradation and phosphorylation remains to be elucidated, as do the identities of the protein kinases involved. We showed that both the N- and the C-termini of SLR1 can be phosphorylated by *EL1* and confirmed that in *el1*, the effects of SLR1 are suppressed (the GA signalling is enhanced) and the GA-mediated degradation of SLR1 is greatly enhanced (Figure 5E). This is consistent with

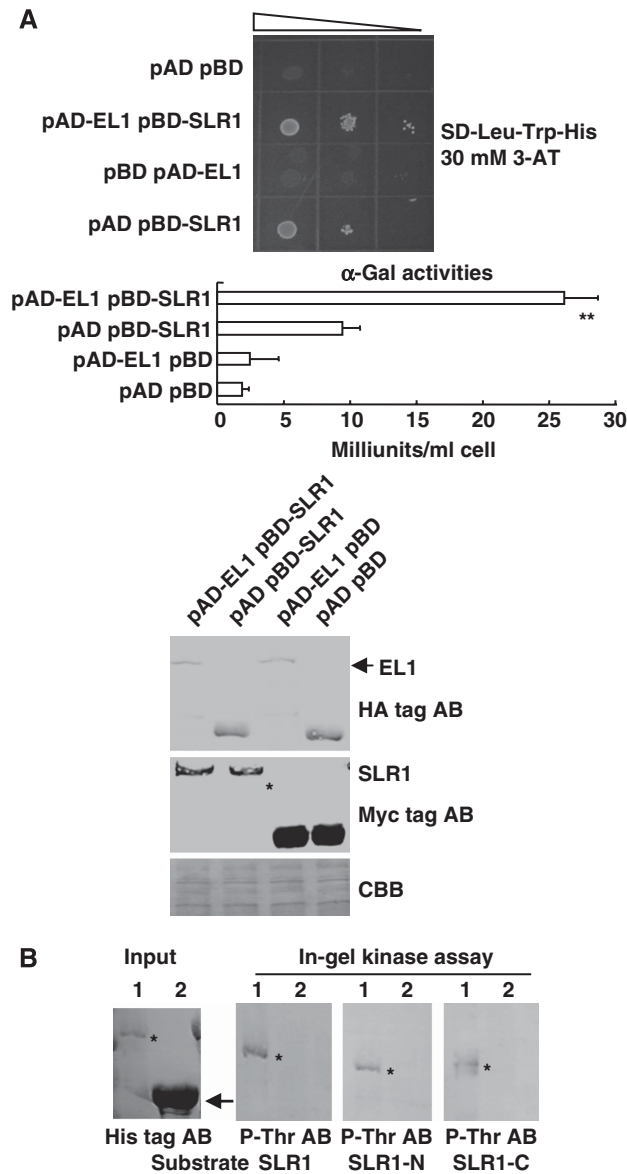


Figure 4 EL1 phosphorylates rice DELLA protein SLR1. (A) Interaction of the EL1 protein with SLR1 (upper panel) and quantitative assay of interactions (bottom panel). Yeast transformants were diluted 10^0 , 10^2 , and 10^4 times and grown on SD-Leu-Trp-His plates (supplemented with 30 mM 3-AT). For the α -galactosidase assay, yeast cells were cultured in liquid medium and values are the mean of two independent assays (10 randomly selected clones were measured each time) and bars indicate the standard deviation (s.d.). Statistical analysis was performed using a heteroscedastic *t*-test (** $P < 0.01$, $n = 10$). Western blot analysis using HA tag or Myc tag antibodies indicated that equal amounts of proteins were used for the assay. (B) In-gel kinase assay revealed that EL1 specifically phosphorylated SLR1 *in vitro*, as well as the N- or the C-terminus of SLR1 (asterisks, recombinantly expressed N- or C-terminus of SLR1 were used as substrate.). The protein extracts of EL1 (lane 1) and OsCKI1 (another member of the rice casein kinase I family, lane 2) were used for the assay. Input of EL1 and OsCKI1 was detected by His tag antibody and the kinase assay was detected by using Thr-P antibody (AB).

the previous reports that the SLR1 N-terminus and C-terminus are responsible for the degradation and activity of SLR1, respectively (Itoh *et al.*, 2002). Constitutive or suppressed phosphorylation of SLR1 at site Ser196 or Ser510 resulted in the suppressed or enhanced GA signalling, which further

confirmed the critical role of phosphorylation of SLR1 on its negative effects in GA signalling. As a sum, these findings indicate that phosphorylation by EL1 is crucial for SLR1 stability at the N-terminus and SLR1 activity at the C-terminus, and hence the regulation of GA responses (Figure 6B). However, phosphorylation of SLR1 by other kinases cannot be excluded, which need further studies and will be helpful to clarify the regulatory mechanism of SLR1 and GA signalling.

In addition, it is worth to notice that the *el1* plants expressing SLR1 are much taller than WT plants expressing SLR1, which nicely correlates with the reduced GA20ox2 and increased GA2ox3 expression in the corresponding lines. However, observation of plant height (Figure 5B) showed that *el1* plants expressing SLR1 is intermediate between WT and WT plants expressing SLR1, and expression levels of GA20ox2 and GA2ox3 are much changed compared to the WT, which may suggest that EL1 has a role in feedback regulation of the GA biosynthetic genes.

Although CKIs modulate the activity of a broad variety of substrates, and multiple isoforms exist in both animals and plants, they have specific roles at certain developmental stages. Concerning the presence of multiple isoforms of CKI in *Arabidopsis* and rice, specific phosphorylation of rice SLR1 by EL1 to modulate the GA response suggests the presence of a specific regulatory mechanism. In addition, distinct domain structures in EL1 compared with those of broccoli (Klimczak and Cashmore, 1993) or yeast (Robinson *et al.*, 1992) further suggest the existence of another regulatory mechanism (aside from the conserved kinase domain and characteristic peptides, EL1 has a more expanded region at the N-terminus).

In summary, the studies presented not only shed light on the mechanisms of GA signalling control, but also provide important clues regarding the mechanism and effects mediated by casein kinase I. CKI is a multifunctional protein kinase involved in hierarchical protein phosphorylation, modulating signal transduction through second-messenger-responsive protein kinases throughout the plant and animal kingdoms. This study will surely expand our knowledge and facilitate a comprehensive understanding of the underlying mechanisms in plants, as well as in animals.

Materials and methods

Isolation of rice early flowering 1 (*el1*) mutant

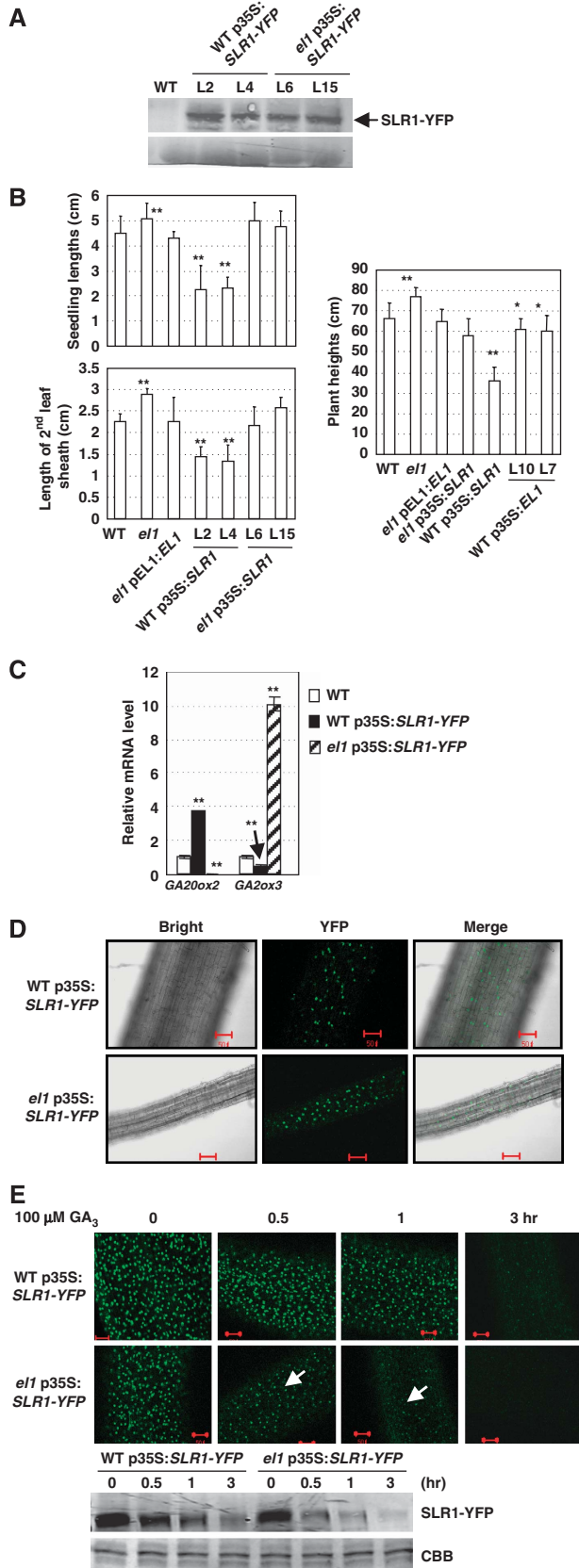
A recessive mutant, *el1*, was identified in rice (*Oryza sativa*, Zhonghua11) T-DNA insertion mutant population (SHIP, <http://ship.plantsignal.cn>, Fu *et al.*, 2009). Surface-sterilized seeds of *el1* mutant and WT plants were soaked in water for 7 days and then placed in soil and grown in a greenhouse. The flanking sequence was obtained by TAIL-PCR (Liu *et al.*, 1995). Primers EL1-1 (5'-CTTCTGGTGTCTCCATTAG-3') and EL1-2 (5'-TGGGAGAGCTGAA GATGTATG-3') were used for PCR amplification to confirm the T-DNA insertion using genomic DNA as template.

Measurement of shoot elongation

Shoot elongation was quantified by a modification of the method described by Matsukura *et al.* (1998). Seeds from WT and *el1* plants ($n = 15-25$) were surface sterilized for 30 min with a 3% NaClO solution, washed four times with sterile distilled water, soaked in the distilled water for 24 h in the presence or absence of different concentration of uniconazole, and then placed in sterile distilled water for another 24 h. The seeds were then placed on a 1% agar plate and grown under fluorescent lamps at 28°C (12 h light/12 h dark). To investigate the role of GA on the elongation of the second leaf sheath, seeds of WT and *el1* mutant plants ($n = 15-25$) were germinated on 1% agar plates supplemented with various

concentrations of GA₃. All plants were grown at 28°C (12 h light/12 h dark) for 7 days. For analysis of surface anatomy, the second leaf sheath was dissected from third-leaf-stage plants and observed using differential interference contrast (DIC) microscopy.

The middles of the second leaf sheath sections were incubated in 9:1 (v:v) ethanol:acetic acid overnight, rinsed with water, and placed in chloral hydrate (glycerol:chloral hydrate:water, 1:8:1, v:w:v). Samples were visualized using a Leica DMR microscope (Germany). The plant heights were measured and calculated when the rice plants had ripened.



Agar plate assay of α -amylase

The agar plate assay was performed essentially as described by Yamachuchi (1998). Seeds were cut transversely and endosperm half-seeds were surface sterilized with 1% NaClO for 15 min, washed with sterile water six times, and placed on 2% agar plates containing soluble starch (0.2%) (Linhu Food Products Factory, China). Plates with 16 or more endosperms were incubated at 30°C in the dark for 48 h, and the endosperms were then removed to examine the α -amylase activity by staining agar with a solution containing 0.1% I₂ and 1% KI.

Protein expression and in-gel kinase assay

The coding region of EL1 was amplified with primers EL1-5 (5'-CATGCCATGGCTATGCCAGAGTTGCGGGT-3', added *Nco*I site underlined) and EL1-6 (5'-GGACTAGTCATACGGTCCGGCCGTAGC-3', added *Spe*I site underlined), and the resultant DNA fragment was sub-cloned into vector pET32c (Novagen, USA). The inducement of recombinant protein expression was carried out by supplement with 1 mM IPTG (28°C, 3 h). The full-length cDNA of SLR1 was amplified with primers SLR1-1 (5'-GGGTACCATGAAGCGCGAGTACCAAGAAG-3', added *Kpn*I site underlined) and SRL1-2 (5'-C GGAATTCGACGCGCCATGCCGAGGTGG-3', added *Eco*R1 site underlined), and then sub-cloned into vector pET32c. The cDNA fragments encoding the N-terminal or C-terminal of SLR1 were amplified with primers SLR-N-1 (5'-CGGAATTCATGAAGCGCGAGTACCAAGAAG-3', added *Eco*R1 site underlined) and SLR-N-2 (5'-CCCAAGCTTGAAGTGGCGAACTTGAGGTAG-3', added *Hind*III site underlined), and SLR-3 (5'-CATGCCATGGAAACCGCAAATCAAGCCATCCTCG-3' added *Nco*I site underlined) and SLR-4 (5'-CGGAATTCGCGGACGCGCCATGCCGAGGTGG-3', added *Eco*R1 site underlined), respectively. The vectors pET32a-AtPIP5K9 (Lou *et al.*, 2007) and pET32a-OsCK11 (Liu *et al.*, 2003) were used for recombination expression of AtPIP5K9 and OsCK11, respectively.

The in-gel kinase assay was performed mainly as described by Murray (<http://www.biocompare.com/protocols/protocol/163/In-Gel-Kinase-Assay.html>) with few modifications. A 10% SDS-PAGE gel containing purified SLR1 protein (0.5 mg/ml) was prepared, and the crude EL1 protein was diluted in 2 × sample

Figure 5 EL1 phosphorylates SLR1 to sustain the activity and stability of SLR1. **(A)** Western blot analysis confirmed the similar levels of SLR1-YFP protein in WT and *el1* plants. Equal amounts of proteins (~10 μg) were used for the blotting and staining by Coomassie brilliant blue (CBB) showed the similar loading of proteins (lower panel). **(B)** Statistical analysis on the lengths of 7-day-old seedlings (left-upper panel), second leaf sheaths (left-bottom panel), and mature plants (right panel), which indicated the negative effects of SLR1 on plant growth were evidently suppressed under *EL1* deficiency. Error bars represent s.d. (*n*=15). Heteroscedastic *t*-test analysis indicated a significant difference (compared with WT, **P*<0.05, ***P*<0.01). **(C)** qRT-PCR analysis revealed that the increased expression of rice *GA20ox2* and suppressed expression of rice *GA2ox3* in WT plants overexpressing *SLR1* were oppositely regulated in *el1* plants overexpressing *SLR1*, indicating the crucial role of EL1 in SLR1 functions. Error bars represent s.d. (*n*=15). Heteroscedastic *t*-test analysis indicated a significant difference (***P*<0.01). **(D)** Phosphorylation of EL1 on SLR1 does not affect the sub-cellular localization of SLR1. Compared with the nuclear localization, deficiency of *EL1* does not alter SLR1 sub-cellular localization. Bar = 50 μm. **(E)** GA-dependent SLR1 degradation was evidently enhanced under *EL1* deficiency under GA₃ treatment (100 μM, for 0.5, 1, or 3 h), revealing the crucial role of EL1 in SLR1 stability. SLR1-YFP fusion protein of 7-day-old WT or *el1* plants (p35S:SLR1-YFP) was observed under a confocal microscope (up panel) and analysed by western blot analysis using GFP antibody (bottom panel, staining CBB showed the similar amounts of loading proteins). Bar = 50 μm.

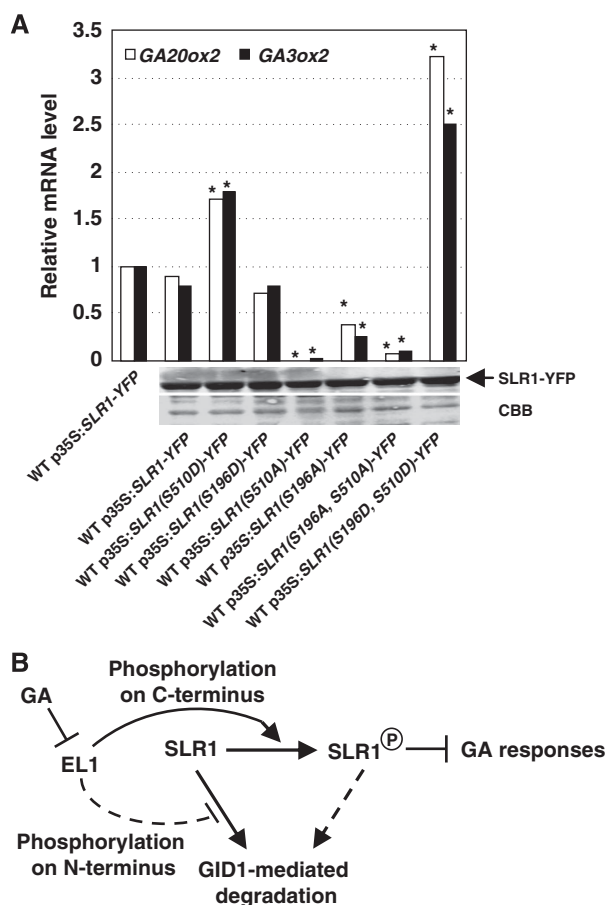


Figure 6 Phosphorylation of EL1 on SLR1 is crucial for GA signalling. (A) After GA₃ treatment (100 μM, 1 h), expressions of *GA20ox2* and *OsGA3ox2* were decreased in WT plants overexpressing mutated SLR1m-YFP (S196A, S510A, and S196A/S510A), which results in the suppressed phosphorylation of these sites, revealing the enhanced GA responses. On the opposite, the expressions of *GA20ox2* and *OsGA3ox2* were increased in WT plants overexpressing mutated SLR1m-YFP (S510D, S196D/S510D), which results in the constitutive phosphorylation of these sites, revealing the suppressed GA responses. qRT-PCR analysis was performed and heteroscedastic *t*-test analysis indicated a significant difference (***P* < 0.01). Expression of the corresponding genes in WT expressing SLR1-GFP without GA₃ treatment was set as 1.0. Western blot analysis confirmed the similar levels of proteins (SLR1-YFP, and SLR1m-YFP) in all of the transgenic lines before GA treatment (bottom panel). (B) Hypothetical model of EL1 function in GA signalling. EL1 is repressed by GA and phosphorylation of EL1 on the SLR1 C-terminus sustains its active form (in red). Phosphorylation on the SLR1 N-terminus suppresses the GID1-GA-mediated degradation of SLR1, resulting in the suppressed GA responses.

buffer [2 mM DTT, 20% glycerol, 100 mM Tris-Cl (pH 6.8), 0.2% bromophenol blue (w/v), 4% SDS (w/v)] and loaded into the gel after boiling for 2 min. After electrophoresis, the gel was washed in wash buffer 1 [50 mM Tris-HCl, pH 8.0, 20% (v/v) 2-propanol] and wash buffer 2 (50 mM Tris-Cl, pH 8.0; 6 M urea; 5 mM 2-mercaptoethanol) each for 1 h at room temperature, then incubated in the wash buffer 3 [50 mM Tris-Cl, pH 8.0; 5 mM 2-mercaptoethanol; 0.04% (v/v) Tween20] at 4°C overnight with gentle rocking. Furthermore, the gel was incubated in the kinase buffer (40 mM Tris-Cl, pH 7.5; 2 mM DTT; 0.1 mM EGTA; 10 mM magnesium acetate) for 30 min at room temperature and then incubated in the buffer containing 20 μM ATP (with rocking) for another 60–90 min. The gel was then transferred to PVDF membrane (PerkinElmer Life Science, USA) by semi-dry blotting and the blot was incubated with rabbit anti-phosphorylation Thr-antiserum (Cell Signaling, USA),

then the bovin anti-rabbit IgG alkaline phosphatase (AP)-conjugated secondary antibody (Santa Cruz, Germany). The AP activity was detected by BCIP/NBT solution (BBI Company, USA).

Semi-quantitative RT-PCR analysis

Total RNAs were extracted and used to synthesize the first-strand cDNAs by reverse transcription. Equal amounts of first-strand cDNAs were used as templates for PCR amplification. Primers EL1-7 (5'-ATGCCAGAGTTGCGGGGTGGT-3') and EL1-8 (5'-AGGTTGATTCCTACACAAATC-3') were used to study the expression pattern of *EL1* in various tissues of WT plants. Primers Hd1-1 (5'-TTCTCCTCTCCAAAGATTCCG-3') and Hd1-2 (5'-AGCAGGTGCAGGATTCTGG-3'); Hd6-1 (5'-TTGTCAGGAAAGTTGGAAGAGG-3') and Hd6-2 (5'-TCCCTGATGATAGAAGTACAGC-3'); *OsMADS1-1* (5'-TGCTCAAGAAGGCCTACGAG-3') and *OsMADS1-2* (5'-TGATGATACC CAATCTGCAGG-3') were used to examine the expression of corresponding genes at different floral stages. The *ACTIN* gene was amplified using primers actin-1 (5'-GAAGTGGTATGGTCAA GGCTG-3') and actin-2 (5'-ACACGGAGCTCGTTGTAGAAG-3') and served as an internal positive control.

qRT-PCR analysis

qRT-PCR analyses were performed to study the transcription of *EL1* in WT (various tissues or under GA₃ treatment), *el1*, and transgenic *el1* plants with complementary expression of *EL1*, or the expressions of genes involved in GA biosynthesis in WT, *el1*, and transgenic WT or *el1* lines overexpressing *SLR1* or mutated *SLR1*. Total RNAs were extracted and used to synthesize cDNAs by reverse transcription. Primers EL1-7 and EL1-8 were used to examine the expression of *EL1*. Primers used to analyse the transcripts of *GA20ox2* (*Sd1*), *GA3ox2*, and *GA2ox3* were as previous description (Sakamoto *et al.*, 2004). The *ACTIN* gene was amplified using primers actin-1 and actin-2, and served as an internal positive control.

EL1-GFP fusion studies

The whole coding region of *EL1* was PCR amplified using primers EL1-5 and EL1-6, and the resultant products were sub-cloned into pCambia1302 vector to generate p35S:EL1-GFP. The construct was sequenced to confirm the in-frame fusion of EL1 to GFP and positive clone was used for transient transformation in onion epidermal cells through particle bombardment (Bio-Rad, USA). Transformed onion cells were culture for 24 h and observed under a confocal microscope (Zeiss LSM 510 META; argon laser excitation wavelength 488 nm for GFP observation).

Constructs and rice transformation

The whole coding region of YFP was amplified with primer YFP-1 (5'-GGACTAGTATGGTGAGCAAGGGCGAGGA, added *SpeI* site underlined) and YFP-2 (5'-GCGTCGACAAGTTGGGTAACCCAGGGT-3', added *SalI* site underlined), and sub-cloned into pBluescript SK(+) (Stratagene, USA). The coding region of *EL1* was amplified with primers EL1-5 and EL1-6, and then sub-cloned into vector pBSK-YFP. The DNA fragment containing EL1-YFP was then sub-cloned into pCambia2301 (Cambia, Australia), resulting in construct p35S:EL1-YFP.

The *EL1* promoter was amplified with primer EL1-9 (5'-ACGGT CGACTGGCATTATCGCCCATGC-3', added *SalI* site underlined) and EL1-10 (5'-AAACTGCAGTACAGTACTAGAGAAATTAC-3', added *PstI* site underlined), and sub-cloned into pBluescript SK(+). The coding region of EL1 was amplified with primers EL1-5 and EL1-6, and then sub-cloned into vector pBSK-pEL1. The DNA fragment containing pEL1-EL1 was then sub-cloned into pCambia2301 (Cambia, Australia), resulting in construct pEL1:EL1.

The full-length cDNA of SLR1 was PCR amplified using primers SLR1-1 and SLR1-4 (5'-GGACTAGTACGCGCCATGCCGAGGTGG-3', added *SpeI* site underlined), and then sub-cloned into pBSK-YFP vector. The fused SLR1-YFP fragment was then sub-cloned into pCambia2301, resulting in the construct p35S:SLR1-YFP.

The partial cDNA of *Hd* genes *Hd1*, *Hd3a*, and *Hd6* were amplified by primers Hd1-3 (5'-ACGCGTCGACCCAGCAGGAGTTG GAGTT-3', added *SalI* site underlined) and Hd1-4 (5'-CGGGATCC GGAGCTGAAGTGAAGGGACA-3', added *BamHI* site underlined); Hd3a-3 (5'-GCTCTAGAGGTTGGTACGGTTGGGGT-3', added *XbaI* site underlined) and Hd3a-4 (5'-GGGTTACCCATGCTGGATGATG ATAGTGCAG-3', added *KpnI* site underlined); Hd6-3 (5'-ACGCGTCCA CGGTGAGCAGGATGACTATGA-3', added *SalI* site underlined) and

Hd6-4 (5'-CGGGATCCTGGAGGAAGTACGGATGTG-3', added *Bam*HI site underlined), respectively. The amplified fragments were sub-cloned into pCambia2301, resulting in constructs p35S:A-Hd1, p35S:A-Hd3a, and p35S:A-Hd6.

The full-length cDNA of SLR1 were amplified by primers SLR1-10 (5'-TCGTCTGCTCATCGTCGGACGACGAC-3') and SLR1-11 (5'-GC GAGGCCCCACCGCCAGGTCGCTGTC-3') for the replacement of Ser196 to Asp; SLR1-9 (5'-AGAGAGCTCGGCCTGGCCGAGTCGCC-3') and SLR1-17 (5'-CCCTCGAGGGCGGCGACTCCGGCCAG-3') were used for replacement of Ser510 to Asp; SLR1-15 (5'-ACGTCGGCAGC AGCAGCAGCAGCAGCA-3') and SLR1-16 (5'-AGGCCCCACCGCCC AGTGCTGCTGCTGC-3') were used for replacement of Ser196 to Ala; SLR1-18 (5'-AGAGAGCTCGGCCTGGCCTGCTGCTGCTGCTC-3') and SLR1-19 (5'-GTTCGATTCCCTCGAGGCGAGCAGCAGCAGGC-3') were used for replacement of Ser510 to Ala. The amplified cDNA fragments were sub-cloned into pBSK-YFP vector, and then the fused SLR1m-YFP fragment was sub-cloned into pCambia2301, resulting in construct p35S:SLR1m-YFP (including S196A, S196D, S510A, S510D, S196A/S510A, and S196D/S510D).

All the positive constructs were introduced into rice WT, *ell* plants, respectively, by *Agrobacterium tumefaciens*-mediated transformation using immature embryos as materials.

Preparation of protein extracts and immunoblot analysis

Total proteins were extracted by grinding rice leaves with liquid nitrogen; the ground tissues were then resuspended in the extraction buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Tween-20, 1 mM EDTA, 1 mM dithiothreitol (DTT)] containing the protease inhibitor cocktail (Roche, Mannheim, Germany). After the addition of an equal volume of 2 × sample buffer [1 × sample buffer: 67.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 0.01% (w/v) bromophenol blue, and 0.1 M DTT], the samples were boiled for 5 min, separated by 8% SDS-PAGE, and transferred to a PVDF membrane by semi-dry blotting. The blots were incubated with mouse anti-GFP antiserum (Neo-Marker, UK) and then with goat anti-mouse IgG AP-conjugated secondary antibody (Santa Cruz, Germany). AP activity was detected by BCIP/NBT solution (BBI Company, USA).

Yeast two-hybrid analysis and α -galactosidase assay

The yeast Gal4 system was used for two-hybrid analysis of EL1 and SLR1 protein interactions. Primers EL1-11 (5'-GATGCCAGAG TTGGGGGTGGT-3') and EL1-12 (5'-CCGCTCGAGGCATACGGTCC GCCCGTAGCAG-3', added *Xho*I site underlined) were used to amplify the EL1 coding region, which was sub-cloned into vector pGADT7 (Clontech, USA). Primers SLR1-11 (5'-GGAATTCATATC AAGCGCGAGTACCAAGAAG-3', added *Nde*I site underlined) and SLR1-12 (5'-CGGAATTCGCCCGCGGCGAGCGCCATGCC-3', added *Eco*RI site underlined) were used to amplify the SLR1 coding region, which was sub-cloned into vector pGBKT7 (Clontech, USA). The yeast strain AH109 was used as the host strain and transformed using a modified lithium acetate method.

The α -galactosidase assay was performed essentially according to the user manual provided by the manufacturers. Ten clones were randomly selected for measurement in each experiment. To confirm that equal amounts of proteins were used for the assay, western blot was performed using extracts prepared from yeast cells as described by Nam and Li (2002). The yeast cells were collected and ground to

a fine powder in liquid nitrogen, and further ground in cold grinding buffer [50 mM HEPES (pH 7.4), 10 mM EDTA, 0.1% Triton X-100, 1 mM PMSF]. After addition of an equal volume of 2 × sample buffer, the samples were boiled for 5 min, separated by 10% SDS-PAGE and transferred to a PVDF membrane by semi-dry blotting. The blots were incubated with mouse anti-Myc antiserum (Neo-Marker, UK), or with rabbit anti-HA antiserum (Santa Cruz, Germany), and then with goat anti-mouse IgG or bovine anti-rabbit IgG AP-conjugated secondary antibody (Santa Cruz, Germany). AP activity was detected by BCIP/NBT solution (BBI Company, USA).

Phylogenetic analysis

Homolog sequences in *A. thaliana*, *O. sativa*, *Saccharomyces cerevisiae*, and *Homo sapiens* were obtained at the NCBI Web site (<http://www.ncbi.nlm.nih.gov/>). The conserved domains of proteins were analysed at the website: <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>. As all casein kinase I homologs had S/T protein kinase domains, we used the conserved domains for phylogenetic analyses. Sequence alignments were generated with CLUSTALX 1.83, and the alignments between *Arabidopsis*, *O. sativa*, *S. cerevisiae*, and *H. sapiens* were adjusted before the tree was constructed. Neighbour-joining analyses were performed using MEGA3 (Kumar *et al.*, 2004) with the pair-wise deletion option, with Poisson correction set for distance model, and 1000 bootstrap replicates selected.

The sequence accession number was obtained from NCBI, as follows: CKIepsilon: AAQ02559 (*H. sapiens*); CKIdelta isoform 2: BAB23405 (*H. sapiens*); CKIbeta isoform: AAS46020 (*Toxoplasma gondii*); CKIalpha (DmCK1): NP_511140; EL1: NP_001051531; CKI: AAA19019; YCK2: NP_014245; Hhp1: NP_595760; OsCKI: CAD32377; AtCK1: NP_193170; AtCK2: NP_188976; CKIgamma 2: AAP36921 (*H. sapiens*); Os01g51200: BAB92346; Os01g13060: NP_001042496; Os01g38950: NP_001043372; Os02g40860: NP_001047465; Os02g56560: EAZ25030; Os02g17910: NP_001046556; Os04g43490: NP_001053309; Os05g51560: NP_001056503; Os10g33650: NP_001064847; CKL3: NP_194617; CKL4: NP_194615; CKL11: AAY24540; CKL9a: AAY24537; CKL2: AAY24533; CKL6: NP_567812; CKL7: NP_199223; CKL1: NP_194340.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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