DWARF3 Participates in an SCF Complex and Associates with DWARF14 to Suppress Rice Shoot Branching

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Introduction

Strigolactones (SLs) are a novel class of plant hormones that inhibit shoot branching. Currently, two proteins in rice are thought to play crucial roles in SL signal transduction. DWARF14 (D14), an α/β hydrolase, is responsible for SL perception, while DWARF3 (D3), an F-box protein with leucine-rich repeats, is essential for SL signal transduction. However, how these two proteins transmit SL signals to downstream factors remains unclear. Here, we characterized a high-tillering dwarf rice mutant, gsor300097, which is insensitive to GR24, a synthetic analog of SL. Mapping and sequencing analysis showed that gsor300097 is a novel allelic mutant of D3, in which a nonsense mutation truncates the protein from 720 to 527 amino acids. The D3 gene was strongly expressed in root, leaf, shoot base and panicle. Nuclear-localized F-box protein D3 played a role in the strongly expressed in root, leaf, shoot base and panicle. The D3 gene was highly conserved in the control of axillary shoot branching. Recently, SL biosynthesis and signal transduction were preliminarily characterized by isolating highly branching mutants from Arabidopsis, rice, pea and petunia. Four genes involved in the biosynthesis of SLs have been identified, and homologous genes were cloned from several species. AtD27/D27 encodes a β-carotene isomerase that converts all-trans-β-carotene into...

SLs and karrikins belong to the butenolide family of small molecules (Zhou et al. 2013). Karrikins are found in the smoke of burned plants and stimulate seed germination to activate the soil seed bank after burning (Nelson et al. 2009, Nelson et al. 2012). Although SLs and karrikins enhance seed germination and promote responses to light, they have distinct functions in the control of shoot branching (Nelson et al. 2009, Mazylis-Gati et al. 2010, Nelson et al. 2010, Yoneyama et al. 2010). GR24 inhibits shoot branching, while karrikins do not suppress axillary shoot growth (Nelson et al. 2011). KAI2, a paralog of DWARF14 (D14), is responsible for karrikin perception (Waters et al. 2012). Both SL and karrikin require MAX2 to function (Nelson et al. 2011). However, how MAX2 transmits different upstream signals remains unclear. MAX2 encodes a 693 amino acid protein containing an F-box domain, an important subunit of the SKP, Cullin, and F-box E3 ubiquitin-protein ligase complex (SCF) (Han et al. 2004, Stirnberg et al. 2007). Targets specifically recognized by the F-box protein are polyubiquitinated and degraded by the 26S proteasome (Gray et al. 1999, Somers and Fujiwara 2009). In the gibberellin signaling mechanism, gibberellin-insensitive dwarf2 (GID2) encodes an F-box protein, which associates with ASK1 and Cullin1 to form an SCF<sub>GID2</sub> complex (Gomi et al. 2004). In the presence of gibberellin, DELLAs were recruited for degradation to generate the gibberellin signal (Dill et al. 2004). In SL signaling, MAX2 participates in the SCF complex by associating with ASK1 and Cullin1 in Arabidopsis (Stirnberg et al. 2007). Whether the rice ortholog DWARF3 (D3) forms an analogous SCF complex remains unknown. In petunia, DAD2 interacts with PhMAX2A based on a yeast two-hybrid assay in a GR24 concentration-dependent manner (Hamiaux et al. 2012). However, the relationship between D3 and D14 in rice requires further characterization.

Here, we characterized the rice high-tillering dwarf mutant gsor300097, which is a novel allelic mutant of D3. Subsequent biochemical analysis showed that D3 assembled into an SCF complex by interacting with OSKs and OsCullin1. Moreover, D3 associated with D14 in a GR24-dependent manner. Our results increase our understanding of SL signal transduction in rice.

**Results**

**Characterization of gsor300097**

The high-tillering dwarf mutant gsor300097 was isolated from the mutagenic population of Akumuro (GSOR300002) using γ-ray radiation (http://ars.usda.gov/Main/site_main.htm?docid=15343). During the young seedling stage, tillers emerged considerably earlier, while shoot length was reduced in gsor300097 compared with the wild type (Fig. 1A). After the heading stage, gsor300097 showed significantly shorter height and significantly increased tiller number compared with the wild type (Fig. 1B). The plant height of gsor300097 was only 50% that of the wild type (Fig. 1C), whereas the tiller number of gsor300097 was 5-fold that of the wild type (Fig. 1D).

**gsor300097 is insensitive to active synthetic strigolactone GR24**

A novel class of phytohormones, the SLs, was recently shown to suppress shoot branching in rice (Gomez-Roldan et al. 2008, Umehara et al. 2008). SL-deficient or -insensitive mutants showed high tillering and a dwarf phenotype, similar to gsor300097. To determine whether gsor300097 was involved in SL biosynthesis or signaling, gsor300097 rice seedlings were treated with GR24, using the SL-deficient mutant gsor300181 (d10<sup>300181</sup>) as a positive control. In the absence of GR24, seedlings sprouted in the tiller buds of 2-week-old gsor300097 and d10<sup>300181</sup>, whereas tiller buds remained dormant in the wild type (Fig. 2A). In the presence of GR24, tiller bud growth was inhibited almost completely in d10<sup>300181</sup>, whereas tiller bud outgrowth of gsor300097 was barely affected by GR24 (Fig. 2A, B). These results demonstrated that gsor300097 is insensitive to GR24, suggesting that gsor300097 has a defect in SL signaling.

**gsor300097 is a novel allelic mutant of D3**

To determine whether the gsor300097 mutant phenotype is controlled by a single gene, we developed an F<sub>2</sub> population between gsor300097 and Guangluai4 (Oryza sativa L. ssp. indica). Of 150 F<sub>2</sub> plants, 32 plants showed high-tillering and dwarf phenotypes. The segregation showed the expected 3 : 1 ratio of a single recessive gene with a χ<sup>2</sup> value of 1.08 (<χ<sup>2</sup> = 3.84), suggesting that the shoot branching phenotype of the gsor300097 mutant was controlled by a single recessive gene.
Expression pattern and subcellular localization of D3

To determine whether D3 is responsible for the high-tillering dwarf phenotype, we performed genetic complementation analysis. The plasmid pCAMBIA1305.1AP-D3-CFH containing the full-length D3 coding sequence driven by the rice ACTIN1 promoter and translationally fused with a FLAG tag at the C-terminus of D3 was introduced into the gsr300097 mutant using Agrobacterium tumefaciens-mediated transformation. A total of 15 independent transgenic lines were obtained, almost all of which complemented the plant height and tiller number phenotype of the gsr300097 mutant based on visual observation. The quantitative plant height and tiller number data of seven independent transgenic lines are provided in Supplementary Fig. S1. Among them, B12-3 and B12-15 showed the best complementation (Fig. 3B–D) and were used for subsequent biochemical analysis.

D3 plays a role in an SCF complex

F-box LRR proteins as a subunit of the SCF-type ubiquitin E3 ligase play crucial roles in plant growth and development.
D3 encodes a 79 kDa protein containing an F-box domain at the N-terminus and LRRs at the C-terminus. Recent studies reported that the F-box domain is an essential element for interaction of F-box proteins with Skp1, Cullin1 and RBX1 to form a Skp/Cullin/F-box complex (Gray et al. 1999, Stirnberg et al. 2007). Therefore, we constructed three bait vectors harboring the full-length D3, the F-box domain or the LRR domain, respectively, to determine whether they interact with these OSKs. Yeast two-hybrid assays showed that the F-box domain (rather than the LRR domain) interacted with OSK1, OSK5 and OSK20, and their interaction was not affected by GR24 (Fig. 5B). However, no interaction was detected between the D3 F-box domain and the other eight OSKs (data not shown). Interestingly, no interaction was detected between full-length D3 and any of the 11 OSKs (Fig. 5B). Full-length D3 protein expressed in yeast may fold incorrectly, or could lack specific modifications or interacting proteins required to expose the F-box domain for interaction with OSKs.

To investigate further whether D3 interacts with OSKs in vivo, we performed co-immunoprecipitation assays in rice protoplasts. FLAG and c-Myc tags were translationally fused to the C-terminus of D3 and the N-terminus of OSKs, respectively. D3-FLAG was driven by the rice ACTIN1 promoter and Myc-OSKs were driven by the Cauliflower mosaic virus (CaMV) 35S promoter. Plasmids pCAMBIA1300.1AP-D3-CFH and pRT105-MYC-OSKs (Myc-OSK1, OSK5, OSK20 or OSK15) were co-transformed into Nipponbare protoplasts. After overnight incubation, total protein was used for immunoblot analysis with anti-FLAG and anti-Myc antibodies. The input protein D3-FLAG and Myc-OSKs were detected in each transformation (Fig. 5C). Anti-c-Myc affinity gels were used to perform immunoprecipitation. After washing, immunoblots were probed with an anti-FLAG antibody. D3-FLAG was pulled-down using Myc-OSK1, OSK5 and OSK20, but not by OSK15 (Fig. 5C), suggesting that D3 interacts with OSK1, OSK5 and OSK20 in vivo.

Cullin is another subunit of the SCF complex. In the rice genome, LOC_Os03g44900 shares the highest homology with Cullin1 in Arabidopsis (therefore we named it OsCullin1). To determine whether OsCullin1 was involved in the D3 complex with OSKs, D3-FLAG and Myc-OsCullin1 or Myc-OSK15 were co-transformed into Nipponbare protoplasts, respectively. D3-FLAG was immunoprecipitated using the anti-FLAG affinity gel. Myc-OsCullin1 was pulled-down by D3-FLAG, while Myc-OSK15 was not (Fig. 5D). Taken together, these results indicated that the F-box protein D3 was involved in an SCF complex with OSK1, OSK5, OSK20 and OsCullin1.

D3 associates with D14 in a GR24-dependent manner

D3 and D14 are important components of SL signal transduction. Recently, it was reported that DAD2 from petunia, an ortholog of rice and Arabidopsis D14, interacts with PhMAX2A in a GR24 concentration-dependent manner in yeast two-hybrid assays (Hamiaux et al. 2012). To determine whether their rice counterparts D3 and D14 interact,
combinations of BD/BD-D3/BD-FB/BD-LRR and AD/AD-D14 plasmids were co-transformed into yeast cells. We did not detect an interaction in these combinations using yeast two-hybrid assays in the presence or absence of 10 μM GR24 (Fig. 6A). To examine whether D3 interacts with D14 in vivo, we employed the T3 homozygous transgenic line B12-15, in which overexpression of the D3-FLAG fusion gene rescued the mutant phenotype (Fig. 3B–D). The pRT105-MYC-D14 construct was transformed into the leaf protoplasts of B12-15. Anti-FLAG affinity gel was used to perform the immunoprecipitation. Myc-D14 was detected by immunoblotting. In the absence of GR24, Myc-D14 was not pulled-down by D3-FLAG, indicating that D3 interacts with D14 in a GR24-dependent manner in plants (Fig. 6B). As a negative control, pRT105-MYC-OSK15 plasmid was also transformed into transgenic plant protoplasts. OSK15 was not pulled-down by FLAG-D3 in the absence or presence of 1 μM GR24 (Supplementary Fig. S2), suggesting that D3 specifically interacts with D14 in the presence of GR24.

Subsequently, we performed a bimolecular fluorescence complementation (BiFC) analysis to confirm whether D3 associates with D14 in vivo. Constructs for expression of the fusion proteins YNE–D3 and YCE–D14 were transiently expressed in the protoplasts from rice seedlings grown on 1/2 Murashige and Skoog (MS) medium with 1 μM GR24.
Different combinations of YNE–D3/pUC-SPYNE and YCE–D14/pUC-SPYCE were co-transformed and the protoplasts were observed under a confocal microscope to detect yellow fluorescent protein (YFP) signal. A strong YFP signal was observed in the nucleus of the protoplast co-transformed with YNE–D3 and YCE–D14 plasmids, whereas no YFP signal was detected in the absence of D3 or D14. This result suggested that the interaction between D3 and D14 occurs in the nucleus (Fig. 6C).

Overexpression of D14 could not complement the \textit{d3}^{300097} mutant phenotype

Auxin receptor TIR1 is an F-box protein containing LRRs and forms an SCF\textsuperscript{TIR1} complex with ASK1/2 and Cullin1 (Gray et al. 2001). Auxin functions by promoting the degradation of the transcriptional repressors Aux/IAA proteins recognized by ubiquitin E3 ligase SCF\textsuperscript{TIR1} (Ruegger et al. 1998, Calderon-Villalobos et al. 2010). MAX2/RMS4/D3/PhMAX2A-2B also encodes an F-box LRR-containing protein and plays a central role in the SL signaling pathway (Wang and Li, 2011, Stanga et al. 2013). To characterize the genetic relationship of D3 and D14, D14 fused with the FLAG tag at its C-terminus (\textit{pCAMBIA1305.1AP-D14-CFH}) was overexpressed in the \textit{d3}^{300097} mutant. The resulting two transgenic lines B117-1 and B117-2 displayed dwarf and high-tillering phenotypes, similar to the \textit{d3}^{300097} mutant (Supplementary Fig. S3), suggesting that the \textit{FLAG-D14} is functional. Immunoblot using anti-FLAG antibody showed that D14 was strongly expressed in B117-1 and B117-2 (Fig. 7B). Thus, \textit{D14} could not complement the high-tillering dwarf phenotype of \textit{d3}^{300097}. This result suggests that D3 may be epistatic to D14, which is consistent with the reports showing that D14 is the SL receptor (Hamiaux et al. 2012, Nakamura et al. 2013).
In this study, we characterized the gsor300097 mutant with short culms and a high-tillering phenotype, similar to the mutants involved in SL biosynthesis or signal transduction. The highly tillering dwarf phenotype of gsor300097 could not be rescued by exogenous GR24, suggesting that gsor300097 is involved in SL signaling. Map-based cloning showed that gsor300097 is a novel allelic mutant of D3, in which the nucleotide G was substituted by A at position 1,583, resulting in formation of a premature stop codon. To determine whether D3 is responsible for the phenotype of d3300097, we expressed the full-length D3 coding region fused with a FLAG tag at its C-terminus driven by the rice ACTIN1 promoter in d3300097. Statistical analysis showed that the plant height of transgenic plants increased...
Fig. 6 D3 associates with D14 in a GR24-dependent manner in vivo. (A) D3 cannot interact with D14 in yeast in the presence of GR24. Yeast strains expressing combinations of BD/BD-D3/BD-FB/BD-LRR and AD/AD-D14 constructs were grown on media minus tryptophan and leucine (left panel), minus tryptophan, leucine and histidine (middle panel), or minus tryptophan, leucine and histidine with 10 μM GR24 (right panel). (B) D3 associated with D14 in a GR24-dependent manner. pRT105-Myc-D14 was transformed into rice leaf protoplasts isolated from 7-day-old transgenic plants containing pCAMBIA1305.1AP-D3-CFH grown in MS medium with or without 1 μM GR24. Anti-FLAG affinity gels were used for immunoprecipitation and an anti-Myc antibody was used to detect D14. (C) D3–D14 interaction analysis using BiFC. Combinations of YNE–D14 and YCE–D3, YNE–D14 and YCE, YNE and YCE–D3 were transiently co-expressed in rice leaf protoplasts. BiFC fluorescence is indicated by the YFP signal. Individual and merged images of YFP and Chl autofluorescence (Chl), as well as bright-field images of protoplasts, are shown. Scale bars = 10 μm.
was transformed into light responses (Shen et al. 2007, Stanga et al. 2013). Axillary shoot growth, delayed leaf senescence and decreased activities were observed in the transgenic plants due to the overexpression of D14 in rice (Fig. 7A). Two transgenic lines, B117-1 and B117-2, were selected for further analysis. The expression of D14 was monitored using antibodies against the FLAG tag. Total proteins extracted from the transgenic plants were analyzed using SDS-PAGE and western blotting. D14 was detected in both B117-1 and B117-2 transgenic lines, indicating the successful overexpression of D14.

To examine the impact of D14 on rice development, several phenotypic assays were performed. The height of transgenic plants was measured, and the data showed that the transgenic plants were taller than the wild type. The number of tillers was also increased in the transgenic plants. The biomass and yield of the transgenic plants were also higher than the wild type, indicating that D14 plays a role in promoting rice growth.

MAX2/D3 encodes an F-box protein that functions as a subunit of the SCF ubiquitin E3 ligase. In an SCF complex, the F-box protein is responsible for specifically recognizing substrates; subsequently, the substrate is marked by polyubiquitination and degraded by the 26S proteasome (Han et al. 2004, Stirnberg et al. 2007). According to the above description, we hypothesized that D3 has multiple substrates in response to different developmental processes. The rice genome contains at least 32 OSK genes (Kong et al. 2007). To identify OSKs that interact with D3, 11 OSK genes from different phylogenetic clades were chosen. Yeast two-hybrid and co-immunoprecipitation assays showed that D3 interacts with OSK1, OSK5 and OSK20. In addition, D3 associated with OsCullin1, the closest ortholog of Cullin1 in Arabidopsis, another subunit of the SCF complex. These results indicated that D3 can associate with OSK and OsCullin1 to form an SCF<sup>D3</sup> complex, and furthermore D3 can form numerous SCF<sup>D3</sup> complexes with different OSKs, although these OSKs may be functionally redundant. These SCF<sup>D3</sup> complexes may be required for D3 to recognize multiple substrates and mediate different developmental processes.

There are considerable similarities between the SL and gibberellin signaling pathways. First, both the gibberellin receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) and the SL receptor D14 belong to the α/β fold hydrolase superfamily (Ueguchi-Tanaka et al. 2005, Hamiaux et al. 2012). Secondly, an E3 ubiquitin ligase complex containing F-box proteins (GID2 or D3) is required for both pathways (Gomi et al. 2004, Stirnberg et al. 2007). During gibberellin signaling, GID1 undergoes a conformational change via gibberellin binding, which stimulates gibberellin–GID1 to interact with the master growth suppressors, the DELLA proteins. DELLA proteins inhibit the function of the gibberellin–GID1–DELLA complex and degrade the DELLA proteins, resulting in the expression of gibberellin signaling (Sun 2011). It has been shown that the F-box protein SLEEPY1 (SLY1), an ortholog of GID2 in Arabidopsis, interacts with the DELLA proteins and associates with the gibberellin receptor GID1b; thus, HA-GID1b can be immunoprecipitated using FLAG-SLY1, and the interaction increased in the presence of gibberellin (Arizumi et al. 2011). During SL signaling, DAD2 from petunia (an ortholog of the rice D14) interacted with F-box protein PhMAX2 (an ortholog of rice D3) in a GR24 concentration-dependent manner based on a yeast two-hybrid assay (Hamiaux et al. 2012). However, we did not detect an interaction between D14 and D3 using the yeast two-hybrid assay, whereas the co-immunoprecipitation and BiFC assays revealed that D3 associated with D14 in the presence of GR24<sup>in vivo</sup> (Fig. 6). Furthermore, we did not detect binding of full-length D3 with the OSK proteins using the yeast two-hybrid assay, although the F-box motif of D3 interacted with OSK proteins (Fig. 5B). These results suggested that the rice D3 protein may not fold correctly in yeast. For the gibberellin signaling mechanism, DELLA suppressors act as a bridge linking GID1b and SLY1 in Arabidopsis, suggesting that proteins analogous to DELLA proteins in the gibberellin signaling pathway may...
be required for the interaction between D3 and D14 during SL signaling. According to previous reports, the Arabidopsis gibberellin biosynthetic mutant ga1-3 showed increased axillary shoot growth (Silverstone et al. 1998). In addition, transgenic plants overexpressing gibberellin 2-oxidase genes in rice showed enhanced shoot branching (Lo et al. 2008). These results suggested that gibberellin regulates shoot branching in an SL-dependent or -independent manner. Recently, the DELLAs protein SLENDER RICE-1 (SLR1) has been shown to interact with D14 in an SL-dependent manner, and the D14–SLR1 interaction competed with the GID1–SLR1 interaction, suggesting that SLR1 may cross-talk between the gibberellin and SL signaling pathways (Nakamura et al. 2013). These results indicated that SLR1 may interact with other DELLAs required for the D3–D14 complex during SL signaling. To date, no DELLA protein analogs downstream of MAX2 have been reported in the SL signaling pathway, although four suppressors of max2 were identified and characterized that function in different developmental processes controlled by MAX2 (Hur et al. 2012, Stirmberg et al. 2012a, Stirmberg et al. 2012b, Stanga et al. 2013).

Recently, a repressor in SL signaling, i.e. D53, was identified, which belongs to the double Clp-N motif-containing P-loop nucleoside triphosphate hydrolase superfamily and acts as a substrate of the SCF3D3 complex. D53 protein was polyubiquitinated and degraded in the presence of active SL analog, GR24. D53 protein accumulated in all d mutants (d10, d17, d27, d3 and d14) because of the lack of SL signaling. Decreased D53 protein levels significantly reduced the tiller number of d3 and d14, suggesting that D53 functions as a key repressor in the SL signaling pathway. D53 interacts with D3 in a GR24-independent manner, while the interaction between D53 and D14 is dependent on the presence of GR24 in a dose-dependent manner (Jiang et al. 2013, Zhou et al. 2013). Therefore, the interaction between D3 and D14 can easily be detected in the presence of GR24, suggesting that D53 may act as an adaptor protein linking D14 and D3 to form a D14–D3–D53 complex.

Materials and Methods

Plant materials and growth conditions

The mutant gsor300097 and wild-type variety GSOR300002 (O. sativa spp. japonica cv. Akumuro) were ordered from USDA-ARS Dale Bumpers National Rice Research Center (http://ars.usda.gov/Main/docs.htm?docid=8318). To examine the phenotypes, wild-type, gsor300097 and transgenic plants overexpressing D3 or D14 were grown in the paddy field in the Experimental Station of Shandong Rice Research Institute, Shandong, China.

Strigolactone treatment

SL treatment was performed as described previously (Umehara et al. 2008), with minor modifications. Briefly, rice seeds were treated with 20% NaClO with 0.1% Triton X-100 in a shaker (150 r.p.m.) for 30 min and then washed four times with sterilized deionized water. Sterilized rice seeds were put on a piece of gauze soaked in water at 28°C in the dark for 2 d. Subsequently, germinated seeds were transferred to Yoshida nutrient solution (Yoshida et al. 1976) with or without 1 μM GR24 (TX23880, Chiralis) and grown in the growth chamber under a 16 h light (28°C)/8 h dark (25°C) cycle for 3 weeks. During this process, the Yoshida nutrient solution was replaced at 2 d intervals.

Plasmid construction

All PCR primers used for plasmid construction are listed in Supplementary Table S2. To overexpress the D3 gene in d300097, the full-length D3 coding region was amplified using PrimeSTAR HS DNA Polymerase with GC Buffer (TAKARA) from the rice seedling cDNA library. PCR products were inserted into the pcambia1305.1AP-CFH vector between PciI and Spel sites, in which the D3-FLAG fusion protein was driven by the rice ACTIN1 promoter (pcambia1305.1AP-D3-CFH). The construct was transformed into the d300097 mutant callus using an Agrobacterium-mediated method.

To monitor subcellular localization of D3, the full-length D3 coding region was inserted into the pcambia1205-GFP vector between XbaI and EcoRI sites, in which the GFP tag was translationally fused to the C-terminus of D3 (pcambia1205-D3-GFP).

To overexpress D14 in the d300097 mutant, D14 cDNA was cloned into pcambia1305.1AP-CFH at the PmlI site, in which the D14-FLAG fusion protein was driven by the rice ACTIN1 promoter (pcambia1305.1AP-D14-CFH) and the resulting vector was transformed into a callus of the d300097 mutant using an Agrobacterium-mediated method.

Quantitative real-time PCR analysis

Total RNA was extracted using TRizol reagent (Invitrogen) from various tissues of rice growing in the paddy field. Total RNA (10 μg) was treated with RNase-free DNase I (TAKARA) to remove contaminating genomic DNA. Subsequently, the resulting 1 μg of RNA was used for reverse transcription with M-MLV reverse transcriptase (TAKARA) and poly(T) primer (TAKARA) according to the manufacturer’s instructions. All the primers used were designed by the SECentral software. The real-time PCR was carried out in a volume of 20 μl containing 0.5 μl of each primer (10 μM), 2 μl of cDNA (12.5 ng μl−1) and 10 μl of 2× SYBR Green PCR master mix (TAKARA). The PCR was performed in the ABI Prism 7300 Sequence Detection System with the programs recommended by the manufacturer (10 s at 95°C and 40 cycles of 95°C for 5 s, 60°C for 20 s and 72°C for 34 s). For each sample, real-time PCR was performed with three independent biological replicates with the following primers: D3, 5’-AACCGGTTTTACCAATTC-3’ and 5’-GCAC CAAGAATCGTCTGGAT-3’; and UBIQUITIN, 5’-AGAAGAGGTCCACCCCTCACC-3’ and 5’-GCATCCCGAAGCTAAACAGCC-3’. UBIQUITIN was used as an internal control. The cycle threshold (Ct) value of the D3 gene was normalized to the Ct value of
the Ubiquitin gene. The relative expression level of the D3 gene was calculated using the 2^−ΔΔCT method (Livak and Schmittgen 2001).

**GUS histochemical assay**

To investigate tissue-specific expression of D3, a 1,672 bp DNA fragment upstream of the translational start codon ATG of D3 was amplified and cloned upstream of the GUS reporter gene in the pCAMBIA1305.1 vector between EcoRI and NcoI sites (pCAMBIA1305.1-D3pro). The construct was transformed into O. sativa japonica var. Nipponbare. Sections from various parts of the transgenic plants were stained with X-gluc (5-bromo-4-chloro-3-indolyl glucuronide, Sigma) as described previously (Jefferson et al. 1987). Briefly, different tissues from transgenic plants were incubated in solutions containing 100 mM NaPO₄ buffer pH 7.0, 2 mM X-Gluc, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.1% Triton X-100 and 10 mM Na₂EDTA at 37°C. Subsequently, the tissues were washed with 75% ethanol several times until they became clear.

**Phylogenetic analysis**

Sequences of OSKs were downloaded from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) under the following accession numbers: OSK1 (LOC_Os11g26910), OSK2 (LOC_Os10g30200), OSK3 (LOC_Os02g13180), OSK5 (LOC_Os09g10260), OSK6 (LOC_Os07g05180), OSK10 (LOC_Os06g20360), OSK15 (LOC_Os08g28820), OSK19 (LOC_Os07g43260), OSK20 (LOC_Os09g36830), OSK21 (LOC_Os07g22680) and OSK28 (LOC_Os07g43240). Amino acid sequences were aligned using ClustalX ver. 1.83. Phylogenetic trees were generated using predicted full-length amino acid sequences using the maximum likelihood method in MEGA6 with bootstrap mode and 500 replications (Tamura et al. 2013).

**Yeast two-hybrid assays**

To detect the interaction between D3 and OSKs, D3 cDNA and the f-box domain at the N-terminus of D3 were amplified and cloned into pGBK7 between Ndel and SalI sites with corresponding primers (Supplementary Table S2), respectively (BD-D3/FB). The LRR domain at the C-terminus of D3 was inserted into pGBKT7 between EcoRI and SalI sites to generate BD-LRR. pGADT7-OSK1, pGADT7-OSK15 and pGADT7-OSK20 were a generous gift from Dr. Cheng Zhukuan of the Institute of Genetics and Developmental Biology, Chinese Academy of Science. Other OSK genes were amplified and cloned into the pGADT7 vector between Ndel and BamHI sites using an InFusion® HD Cloning Kit (TAKARA; AD-OSK genes). The coding sequence of D14 was inserted into the pGADT7 vector between Ndel and BamHI sites (AD-D14). The yeast strain AH109 was used to express pGBK7/TBD-D3/BD-FB/BD-LRR and Y187 was used to express pGADT7/AD-OSKs/AD-D14. Yeast cells were transformed according to the small-scale LiAc yeast transformation procedure. Two-hybrid screening was performed according to the manufacturer’s instructions for the MATCHMARKER Two-Hybrid System (Clontech). The resulting diploid yeasts were selected on synthetic complete medium lacking tryptophan and leucine. Interactions were determined on synthetic complete medium lacking tryptophan, leucine and histidine supplemented with various concentrations of GR24.

**Co-immunoprecipitation assays**

To evaluate the interaction between D3 and OSKs in vivo, the coding region of the OSK genes was cloned into the pRT105-MYC vector at the EcoRI site, in which the c-Myc tag was translationally fused to the N-terminus of the OSK genes (pRT105-MYC-OSKs). In the pCAMBIA1305.1AP-D3-CFH vector, a FLAG tag was fused at the C-terminus of D3. Two constructs were purified with CsCl gradient centrifugation and co-transformed into Nipponbare protoplasts according to a previous protocol (Sheen 2001, Bart et al. 2006). After an overnight incubation, the protoplasts were lysed and centrifuged. Anti-Myc-conjugated agarose (Sigma-Aldrich) was incubated with the extract supernatant for 3 h at 4°C. After washing five times in 1 ml of extraction buffer [10 mM Tris–HCl pH 7.5, 2 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% protease inhibitor cocktail], the co-immunoprecipitation products were detected by immunoblotting. Anti-Myc (Sigma-Aldrich) and anti-FLAG (Sigma-Aldrich) antibodies were used at a 1:3,000 dilution, and chemiluminescence signals were detected using X-ray film.

To investigate the interaction between D3 and OsCullin1, the OsCullin1 coding sequence (LOC_Os03g44900) was cloned into the EcoRI site of the pRT105-MYC vector (pRT105-MYC-OsCullin1). Purified plasmids were transformed into leaf protoplasts of transgenic plants harboring D3-FLAG. An anti-FLAG–agarose conjugate (Sigma-Aldrich) was used for immunoprecipitation.

To explore the association of D3 with D14, the full-length D14 coding region was cloned into the pRT105-MYC vector between BamHI and EcoRI sites (pRT105-MYC-D14). The resulting plasmids were purified and transformed into leaf protoplasts of overexpressed FLAG-D3 transgenic plants grown in solid one-half MS medium with or without 1 μM GR24 for 7 d. Anti-FLAG–agarose conjugate (Sigma-Aldrich) was used in the immunoprecipitation.

**Bimolecular fluorescence complementation (BiFC)**

The coding sequences of D3 and D14 were inserted into the SalI sites of the pUC-SPYCE and pUC-SPYNE vectors to generate YCE-D3 and YNE-D14, respectively (Walter et al. 2004). These plasmids were purified by CsCl gradient centrifugation and transformed into Nipponbare leaf protoplasts (Sheen, 2001, Bart et al. 2006). The YFP fluorescence of protoplasts was assayed 8 h after transformation under a Zeiss LSM 510 META confocal microscope.
Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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