

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

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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 SCF complex by interacting with OSK1, OSK5 or OSK20 and OsCullin1. In addition, D3 associated with D14 in a GR24-dependent manner *in vivo*. Taken together, our findings suggested that D3 assembled into an SCF^{D3} complex and associated with D14 to suppress rice shoot branching.

Keywords: GR24 • High-tillering dwarf • SCF complex • Shoot branching • Strigolactones.

Abbreviations: BiFC, bimolecular fluorescence complementation; D3, DWARF3; D14, DWARF14; GFP, green fluorescent protein; GUS, β -glucuronidase; GID1, GIBBERELLIN INSENSITIVE DWARF1; GID2, GIBBERELLIN-INSENSITIVE DWARF2; InDel, insertion-deletion; LRR, leucine-rich repeat; MS, Murashige and Skoog; OSK, *Oryza sativa* SKP1-like gene; qRT-PCR, quantitative real-time PCR; SCF, SKP, Cullin, F-box E3 ubiquitin-protein ligase complex; SL, strigolactone; SLR1, SLENDER RICE-1; SLY1, SLEEPY1; UTR, untranslated region; YFP, yellow fluorescent protein.

Introduction

Rice is one of the most important cereal crops, which supplies food for more than half of the global population (Wang and Li 2011). The increasing population and decreasing arable land availability make necessary improvement of grain yield per unit area. Rice yield is positively and significantly associated with tiller number per plant, number of grains per panicle and 1000-grain weight (Xing et al. 2002). The rice tiller, which develops from axillary buds in the leaf axils (Minakuchi et al. 2010), is generally considered a key factor in the control of grain yield (Wang and Li 2011). Not all of the axillary buds develop; some continue growing or maintain dormancy, depending on the complex interplay between endogenous signals and environmental factors (Napoli et al. 1999). Until recently, the plant hormones auxin and cytokinin were considered major players in the control of axillary bud growth (Beveridge 2006, Ongaro and Leyser 2008, Minakuchi et al. 2010, Chen et al. 2012). Recently, a novel plant hormone, strigolactone (SL), was discovered to inhibit axillary bud growth (Gomez-Roldan et al. 2008, Umehara et al. 2008).

SLs were first identified for their function in the rhizosphere in both symbiotic and parasitic interactions (Bouwmeester et al. 2007, Yoneyama et al. 2008, de Saint Germain et al. 2013). SLs are derived from the carotenoids and play crucial roles in the control of axillary shoot branching (Umehara et al. 2008, Alder et al. 2012). The SL biosynthesis and signaling pathway is highly conserved in the control of shoot branching in monocots and dicots (Lin et al. 2009, Kebrom et al. 2013). 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

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9-*cis*- β -carotene, which is cleaved by CCD7/MAX3/RMS5/D17/DAD3 and CCD8/MAX4/RMS1/D10/DAD1 to produce carlactone (Sorefan et al. 2003, Booker et al. 2004, Arite et al. 2007, Brewer et al. 2009, Lin et al. 2009, Wang and Li 2011, Alder et al. 2012, Waters et al. 2012). MAX1 encodes a Cyt P450 and acts downstream of carlactone (Scaffidi et al. 2013). These deficient mutants can be rescued by exogenous application of GR24 (Sorefan et al. 2003, Booker et al. 2004, Gomez-Roldan et al. 2008, Umehara et al. 2008). Four genes are involved in SL signal transduction. AtD14/D14/DAD2 encodes an α/β hydrolase, which is thought to function in the perception of SLs (Arite et al. 2009, Gaiji et al. 2012, Hamiaux et al. 2012). MAX2/RMS4/D3/PhMAX2A-2B encodes an F-box leucine-rich repeat (LRR)-containing protein, which plays a central role in SL signal transduction (Ishikawa et al. 2005, Shen et al. 2007, Stirnberg et al. 2007, Beveridge and Kyozuka 2010). BRC1/PsBRC1/FC1 encodes a transcription factor belonging to the TCP family, which may be responsible for downstream output in SL signaling. The phenotype of *d3* can be partially rescued by overexpressing FC1 (Minakuchi et al. 2010). Recently, a dominant SL-insensitive rice mutant *d53* was characterized. D53 encodes a Clp ATPase which is considered a key repressor in SL signaling (Jiang et al. 2013, Zhou et al. 2013).

SLs and karrikins belong to the butenolide family of small molecules (Zhao 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, Mayzlish-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^{GID2} 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*³⁰⁰¹⁸¹) as a positive control. In the absence of GR24, seedlings sprouted in the tiller buds of 2-week-old *gsor300097* and *d10*³⁰⁰¹⁸¹, 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*³⁰⁰¹⁸¹, 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 F₂ population between *gsor300097* and Guangluai4 (*Oryza sativa* L. ssp. *indica*). Of 150 F₂ 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^2 value of 1.08 ($<\chi^2 = 3.84$), suggesting that the shoot branching phenotype of the *gsor300097* mutant was controlled by a single recessive gene.

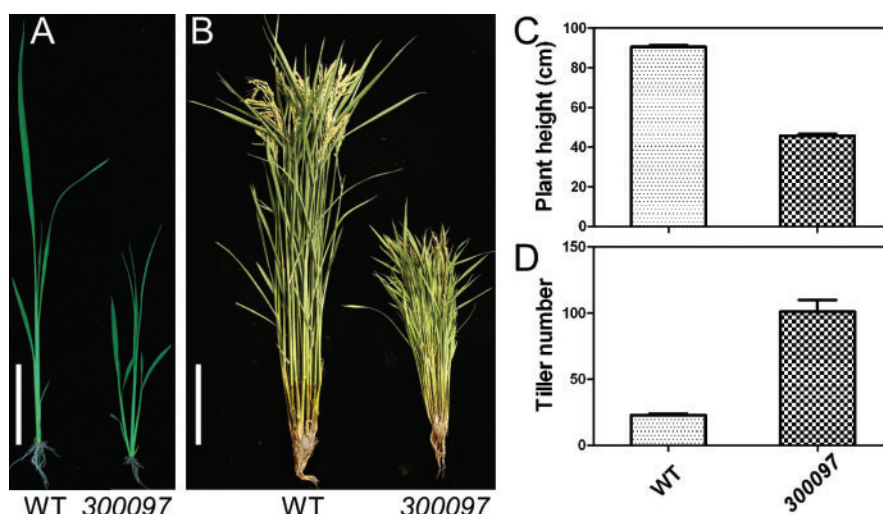


Fig. 1 Phenotype of the *gsor300097* mutant. (A and B) Phenotypic comparison of *GSOR300002* (wild type) and *gsor300097* (300097) at the seedling stage (A) and grain-filling stage (B). Bar = 5 cm in (A) and 20 cm in (B). (C) Plant height of the wild type and *gsor300097* at the grain-filling stage. (D) Tiller number of the wild type and *gsor300097* at the grain-filling stage. In (C) and (D), values represent means $\pm$ SD, $n = 10$.

To identify the mutated gene in the *gsor300097* mutant, we developed five insertion–deletion (InDel) markers tightly linked with the SL-related genes, *D27*, *D10*, *D17*, *D14* and *D3*, respectively (**Supplementary Table S1**). We performed linkage analysis using these markers and 32 recessive plants derived from the F_2 population. We found that the high-tillering phenotype was closely associated with C6-2, an InDel marker for *D3*. Subsequently, the full-length *D3* genomic sequence in the *gsor300097* mutant was sequenced. Position 1,583 in the coding region of *D3* was changed from G to A in the *gsor300097* mutant. This nucleotide change converted amino acid 528 from tryptophan to a premature stop codon (**Fig. 3A**), causing a truncation of the protein from 720 to 527 amino acids. Therefore, *gsor300097* is a new allelic mutant of *D3*, and is named *d3*³⁰⁰⁰⁹⁷ hereafter.

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 *d3*³⁰⁰⁰⁹⁷ 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 *d3*³⁰⁰⁰⁹⁷ 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.

Expression pattern and subcellular localization of *D3*

To evaluate the expression pattern of *D3*, we performed real-time quantitative PCR using mRNA from different organs of *japonica* cv. Nipponbare, including root, culm, leaf, panicle, sheath

and node. The primers used for real-time PCR are located on the third exon of the *D3* gene (**Fig. 3A**). The results showed that *D3* is expressed in different tissues, and the expression of *D3* in panicles, leaves and roots was higher than that in leaf sheaths, internodes and nodes (**Fig. 4A**). This expression pattern was similar to that determined using primers on the first exon of *D3*, but differed from the results obtained using primers on the 3'-untranslated region (UTR), in which *D3* was expressed mainly in the leaves (Ishikawa et al. 2005). This may be because different *D3* transcripts exist in various tissues. To further characterize the expression pattern of *D3*, the *GUS* (β -glucuronidase) reporter gene driven by the 1.67 kb promoter region of the *D3* gene was introduced into the *japonica* cv. Nipponbare. These transgenic lines were used for histochemical assays at different developmental stages (**Fig. 4B–I**). *GUS* activity was strong in the coleoptile and radical areas (**Fig. 4B**). With plant growth, *GUS* activity was also detected in roots, nodes, leaf blades, leaf sheath, tiller buds and panicles (**Fig. 4C–I**). Strong *GUS* activity was observed in roots, the stem base and panicles (**Fig. 4C, H, I**). No *GUS* activity was detected in the root tip of rice (**Fig. 4B, C**). These staining results suggested a pattern of *D3* expression similar to the quantitative real-time PCR (qRT-PCR) analysis.

To explore the subcellular localization of the *D3* protein, we transiently expressed the *D3*–GFP (green fluorescent protein) fusion gene in rice leaf protoplast. After an overnight incubation, the protoplasts were analyzed using a confocal microscope. We found that *D3*–GFP was localized only in the nucleus in rice protoplasts (**Fig. 4G, K**), which is consistent with the nuclear localization of MAX2 in *Arabidopsis* (Shen et al. 2007, Stirnberg et al. 2007).

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

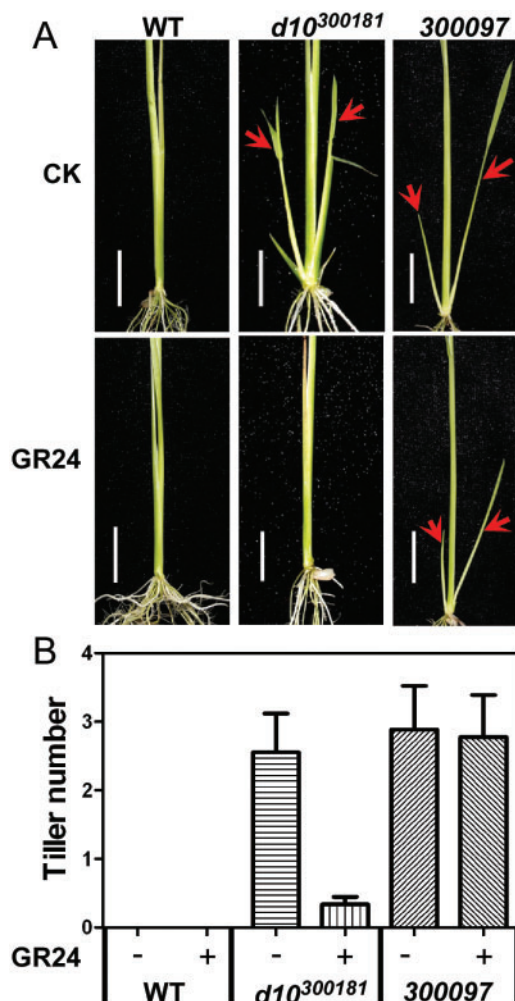


Fig. 2 The *gsor300097* mutant is insensitive to GR24. (A) Response of *gsor300181* (*d10³⁰⁰¹⁸¹*) and *gsor300097* plants to GR24 application. In the absence of GR24, there was no axillary shoot growth in 3-week-old wild-type plants, while the tillers sprouted in *d10³⁰⁰¹⁸¹* and *gsor300097* mutants. The outgrowth of tillers in *d10³⁰⁰¹⁸¹*, but not in *gsor300097*, was suppressed by exogenous GR24. Arrows indicate tiller buds. Bars = 1 cm. (B) Tiller number of 3-week-old wild-type, *d10³⁰⁰¹⁸¹* and *gsor300097* plants treated or not with GR24. Tillers (>2 mm) were counted. Error bars represent the SD ($n = 6$).

(Devoto et al. 2002, Gagne et al. 2002). A canonical SCF complex consists of Skp, Cullin and F-box, which target substrates to the core ubiquitination activity of the complex via direct association with the Skp protein (Gomi et al. 2004, Han et al. 2004). To determine whether D3 participates in an SCF complex in rice, we explored the interaction between D3 and Skps using yeast two-hybrid assays. At least 32 full-length *SKP1* genes [*Oryza sativa* *SKP1*-like genes (OSK genes)] are found in rice (Kong et al. 2007). Eleven OSK genes, i.e. *OSK1*, *OSK2*, *OSK3*, *OSK5*, *OSK6*, *OSK10*, *OSK15*, *OSK19*, *OSK20*, *OSK21* and *OSK28*, belonging to different gene clades based on phylogenetic analysis were tested for an interaction with D3 (Fig. 5A). 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 *pCambia1305.1AP-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,

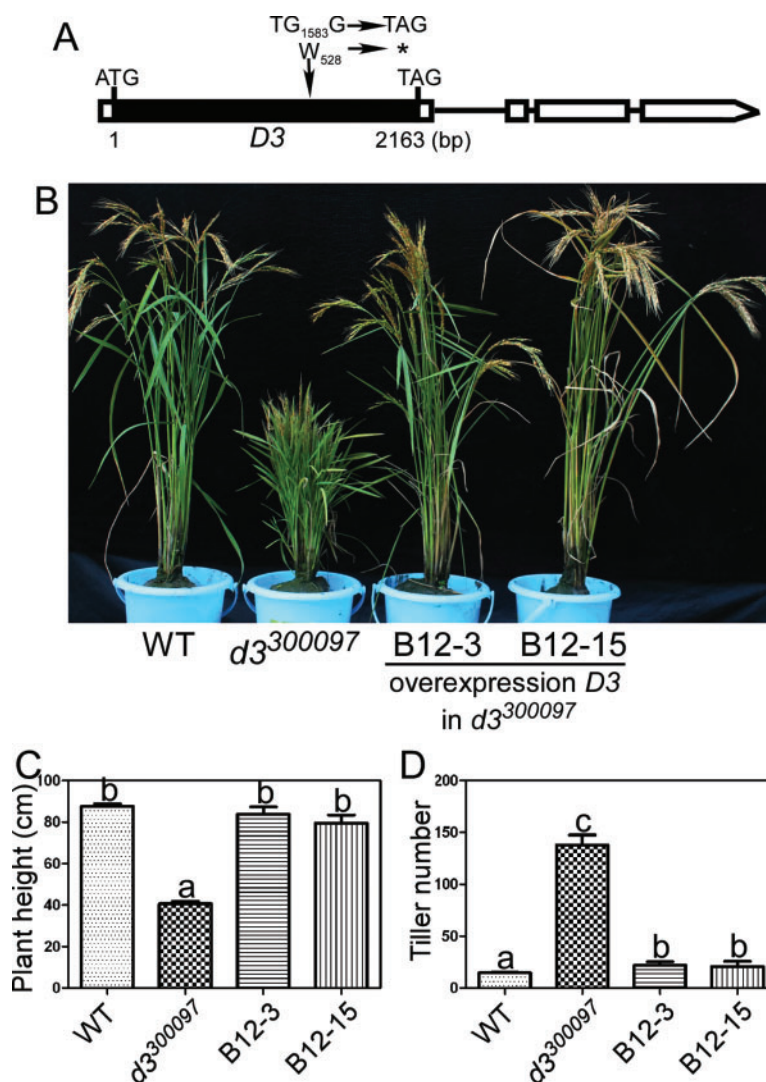


Fig. 3 Mutation site of D3 in *gsr300097* and phenotypic analysis of transgenic plants overexpressing D3 in *gsr300097*. (A) Schematic representation of the D3 gene. Nucleotide position 1,583 in the D3-coding sequence was changed from G to A in *gsr300097*, resulting in a premature stop codon. (B) Phenotype of transgenic plants overexpressing D3 in *dsr300097*. B12-3 and B12-15 are two representative lines. (C) and (D) Plant height (C) and tiller number (D) of transgenic plants overexpressing D3 in *dsr300097*. The culm length of transgenic plants was recovered to wild-type levels, whereas the tiller number increased slightly compared with the wild type. Student's *t*-test was used to determine statistical significance; significant differences ($P < 0.05$) are indicated by different lower case letters. $n = 10$.

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 μ M 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 or the quantity of Myc-D14 immunoprecipitated by D3-FLAG was below the level of detection. Interestingly, in the presence

of 1 μ M GR24, Myc-D14 was 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 μ M 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 μ M GR24.

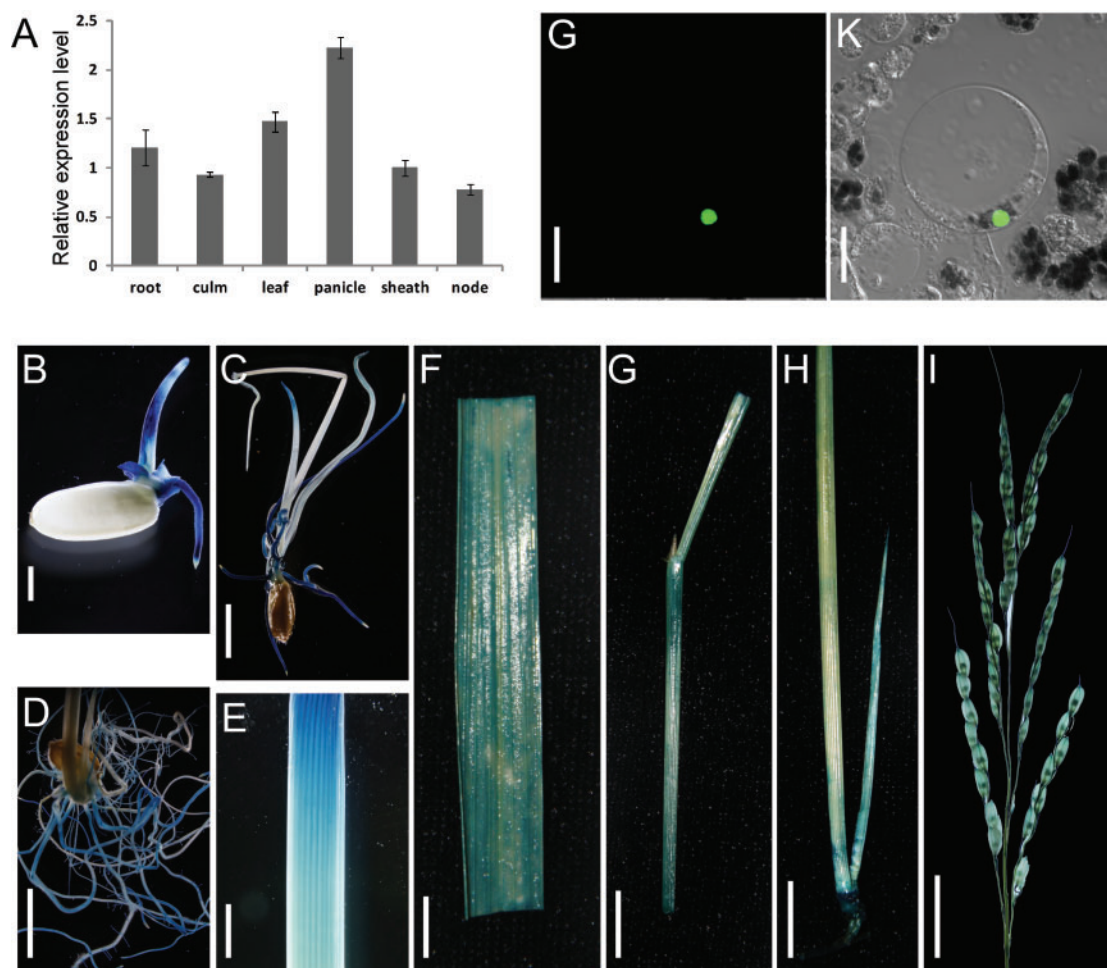


Fig. 4 Expression pattern of D3 and subcellular localization of the D3 protein. (A) D3 expression levels in various organs, including roots, culms, leaves, panicles, sheaths and nodes, were determined using qRT-PCR. Values represent means $\pm$ SD of three independent experiments. (B–I) D3 expression was detected by histochemical GUS staining. D3 was strongly expressed in roots (B–D), culm (E), leaf blade (F), leaf sheath (G), shoot base (H) and panicle (I). Scale bars = 0.2 cm in (D) and (E); 0.6 cm in (B) and (C); 1 cm in (F); 2 cm in (G) and (H). (J and K) D3 localized in the nucleus. Scale bars = 100 μ m.

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 *d3*³⁰⁰⁰⁹⁷ mutant phenotype

Auxin receptor TIR1 is an F-box protein containing LRRs and forms an SCF^{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^{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 (*pCAMBIA1305.1AP-D14-CFH*) was overexpressed in the *d3*³⁰⁰⁰⁹⁷ mutant. The resulting two transgenic lines B117-1 and B117-2 displayed dwarf and high-tillering phenotypes, similar to the *d3*³⁰⁰⁰⁹⁷ mutant (**Fig. 7A**). However, the same construct rescued the *d14* mutant phenotype (**Supplementary Fig. S3**), suggesting that the FLAG-D14 is functional. Immunoblot using anti-FLAG antibody showed that D14 was strongly expressed in B117-1 and B117-2 (**Fig. 7B**). Thus, D14 could not complement the high-tillering dwarf phenotype of *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).

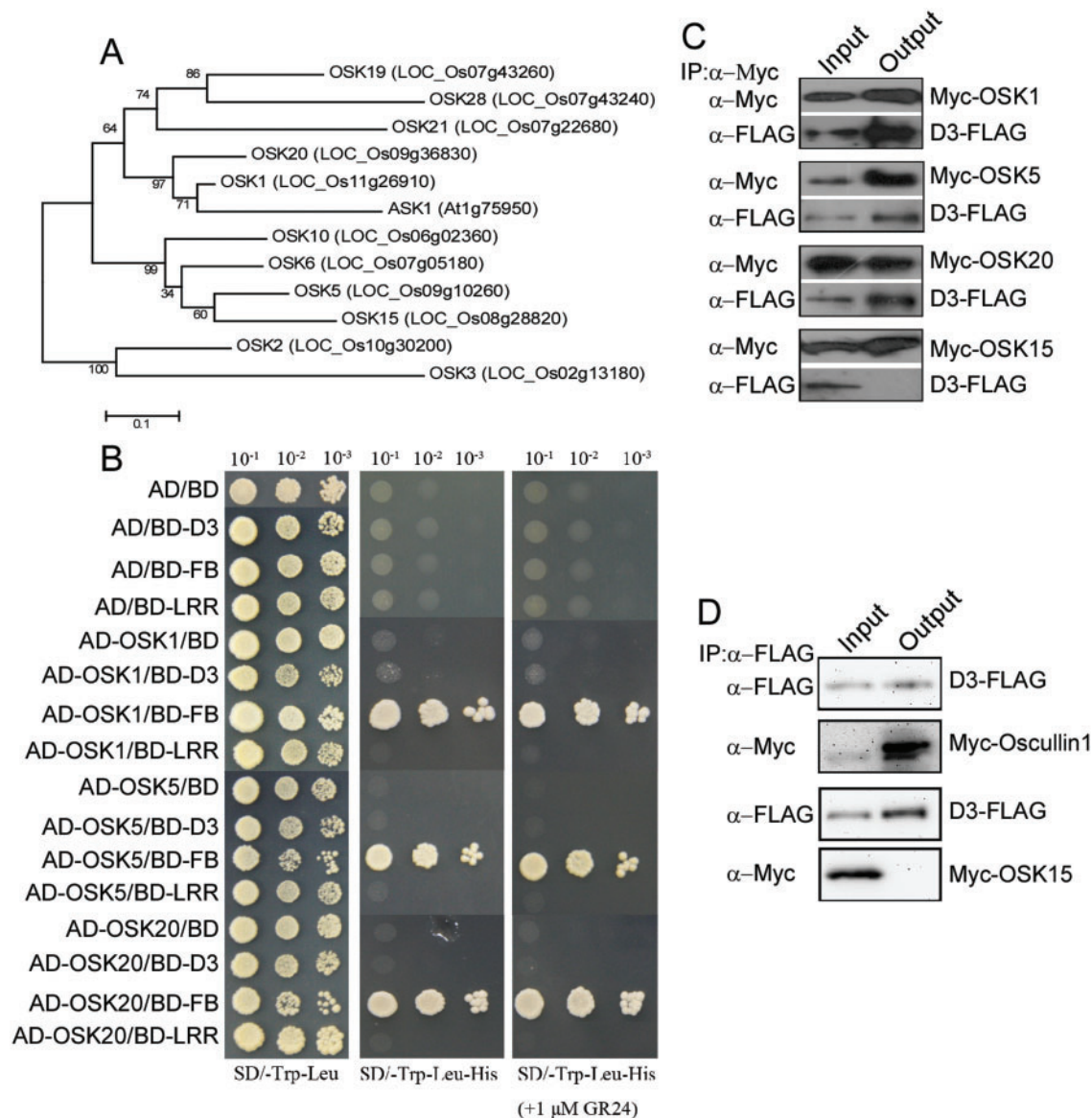


Fig. 5 D3 participated in an SCF complex associated with different OSKs and OsCullin1. (A) Phylogenetic analysis of ASK1 and 11 OSKs. The tree was generated using predicted full-length amino acid sequences using the maximum likelihood method in MEGA6 in bootstrap mode with 500 replications. (B) The F-box domain of D3 interacted with OSK1, OSK5 and OSK20 in yeast two-hybrid assays. Yeast strains expressing the indicated 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 1 μ M GR24 (right panel). (C) D3 interacted with OSK1, OSK5 and OSK20 *in vivo*. Plasmids *pRT105-Myc-OSKs* and *pCAMBIA1305.1AP-D3-CFH* were co-transformed into rice leaf protoplasts. Anti-Myc affinity gel was used for immunoprecipitation and an anti-FLAG antibody was used to detect D3. (D) D3 was associated with OsCullin1 *in vivo*. The *pRT105-Myc-OsCullin1* plasmid was transformed into rice leaf protoplasts isolated from B12-15. Anti-FLAG affinity gels were used for immunoprecipitation, and an anti-Myc antibody was used to detect OsCullin1.

Discussion

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

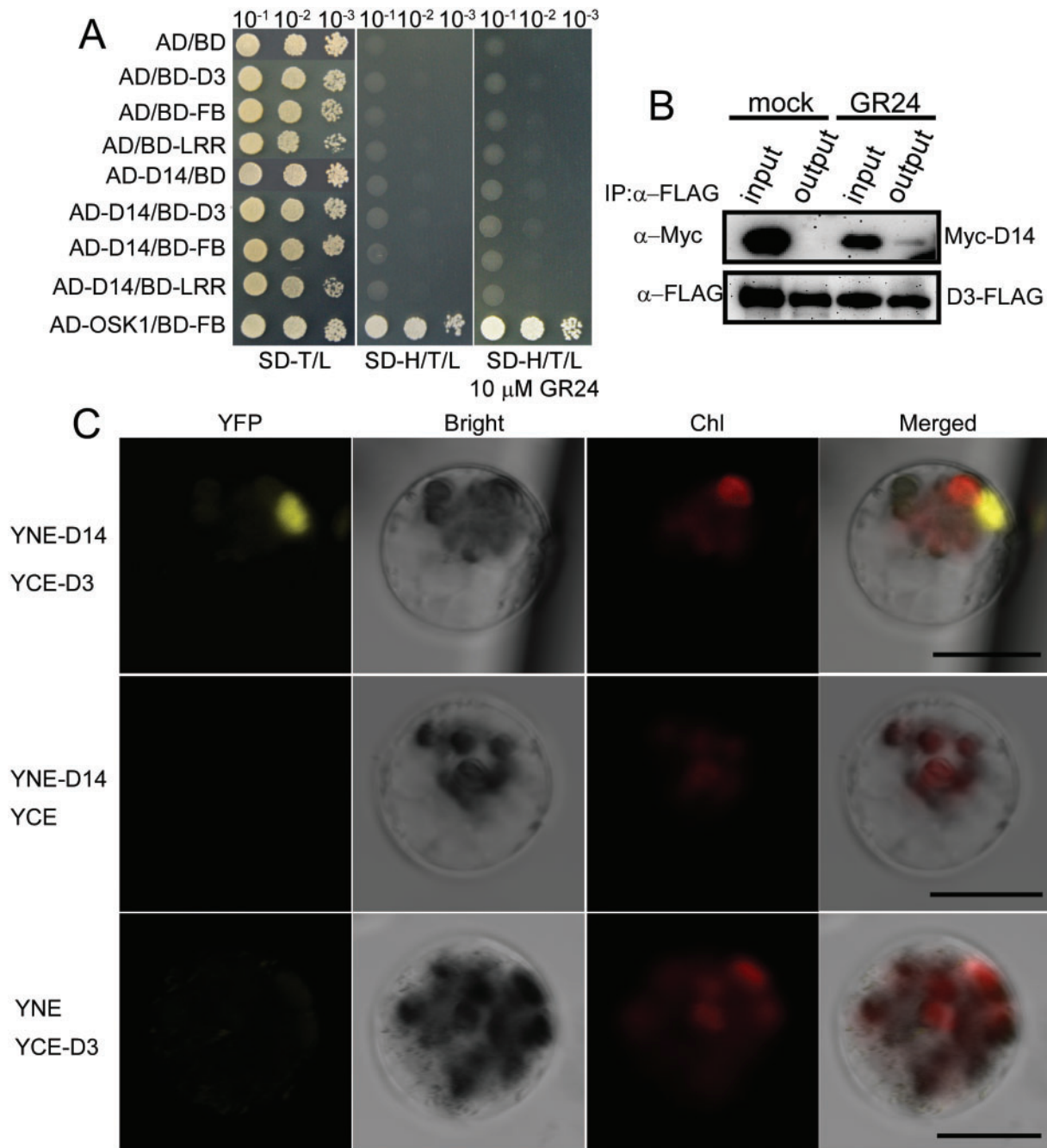


Fig. 6 D3 associates with D14 in a GR24-dependent manner *in vivo*. (A) D3 cannot interact with D14 in yeast in the presence of GR24. Yeast strains expressing combinations of BD/BD-D3/BD-FB/BD-LRR and AD/AD-D14 constructs were grown on media minus tryptophan and leucine (left panel), minus tryptophan, leucine and histidine (middle panel), or minus tryptophan, leucine and histidine with 10 μ M 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 μ M 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 μ m.

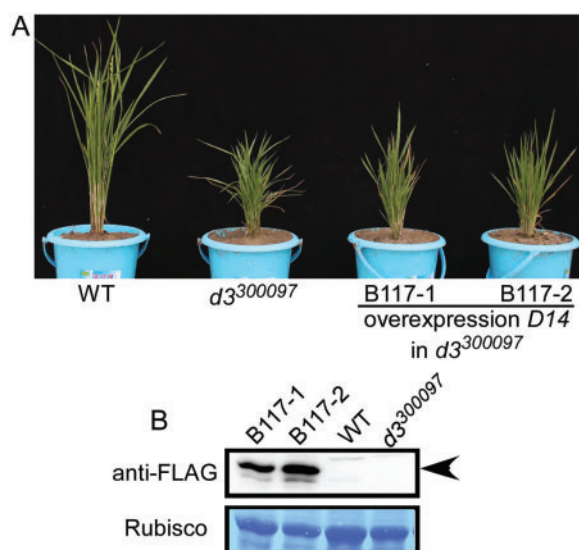


Fig. 7 Phenotypic analysis of transgenic plants overexpressing D14 in $d3^{300097}$. (A) The phenotype of B117-1 and B117-2 transgenic lines overexpressing D14 in $d3^{300097}$. Plasmid *pCambia1305.1AP-D14-CFH* was transformed into $d3^{300097}$ to generate B117 transgenic lines. Two of 24 transgenic lines were photographed. (B) D14 expression in B117-1 and B117-2. Total proteins (10 μ g) extracted from the transgenic plants at the tillering stage were used to detect the expression of D14. Rubisco was used as a loading control.

almost to the level of the wild type, while the tiller number was slightly higher than that of the wild type (Fig. 3B, C). This may be because the *ACTIN* promoter cannot represent the native promoter of D3, or the FLAG tag at the C-terminus of D3 partially affects the function of D3. In addition, the rice genome annotation project website (<http://rice.plantbiology.msu.edu/>) predicts that D3 contains a long UTR at its 3' end. The constructs used for complementation did not contain the 3'-UTR sequence of D3, suggesting that the 3'-UTR may be required for D3 function in controlling shoot branching.

D3 encodes a 720 amino acid protein with an F-box motif at its N-terminus and 18 LRRs at its C-terminus (Ishikawa et al. 2005). In the $d3^{300097}$ mutant, the substitution of G by A at position 1,583 results in a premature stop codon. The resultant truncated protein of 527 amino acids contains an F-box motif and 13 intact LRRs. The F-box motif is required for the interaction with OSKs to form an SCF complex, and LRRs function in the recruitment of substrates for the SCF complex for subsequent ubiquitination (Ishikawa et al. 2005). In the $d3^{300097}$ mutant, the substrate(s) may not be recruited and degraded by the SCF^{D3} complex due to lack of the last five LRRs. The accumulated substrates may block SL signal transduction and cause the high-tillering dwarf phenotype.

MAX2 plays a crucial role in regulating SL and karrikin signal transduction and regulates a broad range of developmental processes. The *Arabidopsis max2* mutant showed increased axillary shoot growth, delayed leaf senescence and decreased light responses (Shen et al. 2007, Stanga et al. 2013).

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^{D3} complex, and furthermore D3 can form numerous SCF^{D3} complexes with different OSKs, although these OSKs may be functionally redundant. These SCF^{D3} 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. DELLAs in the gibberellin-GID1-DELLA complex can be recognized by the F-box protein GID2, subsequently polyubiquitinated by the SCF^{GID2} complex and degraded by the 26S proteasome, resulting in 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 (Ariizumi 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 *in vivo* (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 DELLAs 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 DELLA 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 D14 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, Stirnberg et al. 2012a, Stirnberg 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 SCF^{D3} 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 μ M GR24 (CX23880, Chiralix) 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 *d3*³⁰⁰⁰⁹⁷, 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 *SpeI* 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 *d3*³⁰⁰⁰⁹⁷ 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 *d3*³⁰⁰⁰⁹⁷ mutant, D14 cDNA was cloned into *pCambia1305.1AP-CFH* at the *PmII* 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 *d3*³⁰⁰⁰⁹⁷ 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 μ g) was treated with RNase-free DNase I (TAKARA) to remove contaminating genomic DNA. Subsequently, the resulting 1 μ g 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 μ l containing 0.5 μ l of each primer (10 μ M), 2 μ l of cDNA (12.5 ng μ l⁻¹) and 10 μ l of 2 $\times$ 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'-AAGCCGGTTTATCCAATTCC-3' and 5'-GCACCAAGAATCGTCTGGAT-3'; and *UBIQUITIN*, 5'-AGAAGGAGTCACCCCTCCACC-3' and 5'-GCATCCAGCACAGTAAAACACG-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 $2^{-\Delta\Delta CT}$ 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_Os06g02360), 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 *pGBKT7* between *NdeI* and *Sall* 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 *Sall* 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 *NdeI* and *BamHI* sites using an In-Fusion[®] HD Cloning Kit (TAKARA; AD-OSK genes). The coding sequence of *D14* was inserted into the *pGADT7* vector between *NdeI* and *BamHI* sites (*AD-D14*).

The yeast strain AH109 was used to express *pGBKT7/BD-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 *Sall* 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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