Rice microtubule-associated protein IQ67-DOMAIN14 regulates grain shape by modulating microtubule cytoskeleton dynamics

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Summary
Cortical microtubule (MT) arrays play a critical role in plant cell shape determination by defining the direction of cell expansion. As plants continuously adapt to ever-changing environmental conditions, multiple environmental and developmental inputs need to be translated into changes of the MT cytoskeleton. Here, we identify and functionally characterize an auxin-inducible and MT-localized protein OsIQ67-DOMAIN14 (OsIQD14), which is highly expressed in rice seed hull cells. We show that while deficiency of OsIQD14 results in short and wide seeds and increases overall yield, overexpression leads to narrow and long seeds, caused by changed MT alignment. We further show that OsIQD14-mediated MT reordering is regulated by specifically affecting MT dynamics, and ectopic expression of OsIQD14 in Arabidopsis could change the cell shape both in pavement cells and in hypocotyl cells. Additionally, OsIQD14 activity is tightly controlled by calmodulin proteins, providing an alternative way to modify the OsIQD14 activity. Our results indicate that OsIQD14 acts as a key factor in regulating MT rearrangements in rice hull cells and hence the grain shape, and allows effective local cell shape manipulation to improve the rice yield trait.

Introduction
Rice (\textit{Oryza sativa}) is one of the most important food crops for nearly half of the world population. Control of plant shape and architecture has been a major topic in rice plants and has great potential for crop yield improvement (Gupta et al., 2006). Specifically grain size, controlled by the length and width of the grain, is a key factor determining final yield and is mainly restricted by spikelet hull size (Zuo and Li, 2014). Many genes controlling grain size have been identified in rice, and recent studies have shown that this trait is controlled by multiple signalling pathways involving phytohormones, G-proteins and ubiquitin-mediated proteasomal degradation pathways (Li and Li, 2016; Zuo and Li, 2014). Despite these advances, it remains largely unknown how these pathways control the spikelet hull size at cellular level.

Microtubule (MT) dynamics and the cytoskeleton in general play an important role in plant morphogenesis (Hashimoto, 2015). Ordered cortical MT arrays play a critical role in plant cell shape formation by defining the axis of cell expansion through control of cellulose orientation and cell wall organization. As a result, MT organization determines directional cell expansion and plant morphogenesis (Baskin, 2001; Gutierrez et al., 2009; Paredez et al., 2006). However, due to pleiotropic effects upon MT modification, it is challenging to exploit MT array organization for crop improvement. Many MT-associated proteins (MAPs) play vital roles in organizing the MT cytoskeleton and cell shape formation (Hashimoto, 2015). Nevertheless, how these proteins cooperatively regulate MT dynamics, and how they integrate multiple environmental and developmental signals to control cell morphogenesis and plant shape, remains a major unanswered question in plant biology.

Recently, the plant-specific IQ67-DOMAIN (IQD) family emerged as regulators of fruit shape and grain size in several plant species including tomato (Xiao et al., 2008), watermelon (Duo et al., 2018), cucumber (Pan et al., 2017) and rice (Duan et al., 2017; Liu et al., 2017). For example, the tomato SUN gene encodes a member of IQD family and is involved in regulating fruit shape. Increased SUN expression led to elongated tomato shape (Xiao et al., 2008). Similar elongated fruit shape was found in watermelon and cucumber, associated with high IQD expression, suggesting that a conserved mechanism may exist for IQD-mediated fruit shape regulation. However, their precise biological roles and biochemical functions remain to be elucidated, especially in crop plants. IQD proteins contain a conserved, 67 amino acid domain and were first identified in Arabidopsis where it is represented by a 33-member gene family (Abel et al., 2005). Evidence form Arabidopsis showed that IQDs play important roles in leaf shape determination and xylem secondary cell wall architecture (Burstenbinder et al., 2017; Liang et al., 2018; Sugiyama et al., 2017). Most of the IQD genes have distinct expression patterns, preferentially in embryo, stomata, meristematic cells and elongating tissues (Burstenbinder et al., 2017; Wendrich et al., 2018). Previous studies also showed that IQD proteins can associate with microtubules or the plasma...
Results

_OsIQD14_ is Auxin-inducible and transcribed during seed hull development

Considering that the _Arabidopsis_ AtIQD15-18 subclade, acting downstream of the Auxin RESPONSE FACTORS/MONOPTEROS (ARF5/MP) transcription factor (Möller et al., 2017), is represented by a single _OsIQD14_ ortholog in rice (Abel et al., 2005), we analysed its expression pattern using the transcript digital gene chip. We found _OsIQD14_ to be highly expressed in inflorescences, pistils and spikelet hull tissues (Figure S1a), indicating that _OsIQD14_ may have potential roles in grain size regulation. Quantitative RT-PCR (qRT-PCR) analysis further showed that _OsIQD14_ transcripts can be detected during panicle development, peaking around the middle stage and gradually decreasing in mature stages of development, confirming _OsIQD14_ is expressed highly in the inflorescence (Figure 1a).

We further analysed the expression pattern by fusing the _OsIQD14_ promoter region to the β-glucuronidase (GUS) reporter. Young spikelet hulls and anthers showed a strong GUS signal (Figure 1b-d), indicating that _OsIQD14_ may play a role during rice spikelet hull development. As some of the _Arabidopsis_ orthologs were reported to be auxin-inducible (Möller et al., 2017), we analysed _OsIQD14_ transcript levels upon exogenous auxin treatment. qRT-PCR analysis confirmed that _OsIQD14_ transcripts were quickly induced upon auxin (indole 3-acetic acid, IAA) treatment (Figure 1e), suggesting that _OsIQD14_ may also be transcriptionally regulated by ARFs in rice.

_OsIQD14_ controls grain dimensions by regulating spikelet hull cell shape

To define the biological function of _OsIQD14_, we generated loss-of-function mutants by using CRISPR/Cas9 technology (Miao et al., 2013). A construct expressing a guide RNA targeting the first exon of _OsIQD14_ was transformed into ZH11 wild-type (WT) rice plants. Five independent homozygous lines were isolated that carried frame shift mutations resulting from a 1-bp (_iqd14-1_) insertion or a 5-bp (_iqd14-2_), 22-bp (_iqd14-3_), 34-bp (_iqd14-4_) or 3-bp (_iqd14-5_) deletion, respectively (Figure S1b). None of the independent mutants presented a visible phenotype during vegetative growth, but four of them (except the 3-bp deletion line _iqd14-5_) produced wider and shorter grains compared to those of ZH11 (Figures 2a-b, d-e and 3c, d), demonstrating a role for _OsIQD14_ in panicle and spikelet development. The 3-bp deletion line ( _iqd14-5_ ) results in a single amino acid deletion without visible phenotypes, suggesting that the phenotype observed in other four frame shift mutations is not due to off-target effects. For all further analyses, the 5-bp deletion _iqd14-2_ mutant allele (labelled as _iqd14-C_) was selected.

To determine whether _OsIQD14_ was not only necessary, but also sufficient for regulating grain shape, we next generated overexpression plants by driving _OsIQD14_ from the strong p35S promoter in a ZH11 background (p35S::_OsIQD14::GFP). In contrast to the ZH11 and _iqd14-C_ mutant, plants overexpressing _OsIQD14_ produced narrower and longer grains (Figure 2c, f and g-h). Furthermore, the 1000-grain weight of _iqd14-C_ was significantly increased compared to that of ZH11, while that of p35S::_OsIQD14::GFP was similar to ZH11 (Figure 2i). Importantly, the panicle of _iqd14-C_ and p35S::_OsIQD14::GFP was similar to that of ZH11 (Figure 2j).

As the spikelet hull has been proposed to restrict growth of a grain and as such to determine grain size, we examined epidermal morphology of individual spikelet hull cells in the outer and inner glume of the lemma of ZH11, _iqd14-C_ and p35S::_OsIQD14::GFP plants. Scanning electron microscopy (SEM) analysis revealed that _iqd14-C_ spikelet hull cells were shorter and wider than that of ZH11 (Figure 3a-b, d-e, g-h and j-k). Conversely, the hull cells of p35S::_OsIQD14::GFP plants were narrower and longer than those of ZH11 and _iqd14-C_ plants (Figure 3c, f, i, l). Although _iqd14-C_ showed a decreased hull cell length and p35S::_OsIQD14::GFP plants presented promoted hull cell elongation (Figure 3m), they both showed decreased hull cell numbers (Figure 3n), suggesting the altered seed shape is due to the hull cell shape alterations, but not due to _OsIQD14_-mediated cell division control. Further counting the cell numbers in the spikelet hull of _iqd14-C_ and p35S::_OsIQD14::GFP plants in the grain-width direction confirmed that there was no difference compared to that of ZH11 (Figure 3o). Taken together, these results indicate that the changes in overall seed shape by modifying _OsIQD14_ levels (e.g. short and wide versus long and slender) are caused by the same type of changes to the individual spikelet hull cells, and that modifying _OsIQD14_ transcript levels can be used as a tool to modify rice grain shape without a yield penalty under normal growth conditions.

_OsIQD14_ is a microtubule-binding protein

To understand through what mechanism _OsIQD14_ can alter the cell shape, we first analysed its subcellular protein localization. Similar to some IQD proteins in _Arabidopsis_ (Burstenbinder et al., 2017; Burstenbinder et al., 2013), _OsIQD14::GFP_ fusion protein is localized to cytoskeleton-related structures in rice root cells (Figures 4a and 5d) and leaves cells (Figure 4b, c). Similar results were observed by transiently expressing _OsIQD14::GFP_ fusion protein in _Nicotiana benthamiana_ epidermal cells (Figure 5a). Considering that the cytoskeleton-associated localization was abolished by application of the MT depolymerization drug oryzalin (Figure 4d-f), _OsIQD14_ thus likely localizes to MT. To better understand this MT-associated localization, we used super resolution Structured Illumination Microscopy (SIM) in _Arabidopsis_ root meristem cells. Intriguingly, we observed the punctate localization of _OsIQD14_ along MTs (Figure 5b), while general MT-associated proteins (p35S::MAP4::GFP, Mei et al., 2012) showed uninterrupted distribution along the filaments (Figure 5c). This result suggests that _OsIQD14_ is not a generic MT-binding protein, but associates with MT at specific positions.
OsIQD14 expression of (e) qRT-PCR analysis revealed the up-regulated expression in young panicle (0.4-cm in length) and lemma before anthesis of rice. Representative images are shown. Bar = 2 mm.

Promoter-GUS fusion analysis showed the OsIQD14 expression in young panicle (0.4-cm in length) and lemma before anthesis of rice. Representative images are shown. Bar = 2 mm.

(qRT-PCR) analysis revealed the up-regulated expression of OsIQD14 under short time (30–420 min) auxin treatment (10 µM IAA). Rice seedling roots were used, expressions were normalized with the ACTIN transcript, and relative expression levels were calculated by setting the OsIQD14 expression in the absence of auxin as ‘1.0’. Experiments were biologically repeated, and data are shown as mean ± standard error (SE).

To examine whether OsIQD14 interacts with MT directly, we performed a MT spin-down assay. Similar to MAP2, a generic MT-binding protein and positive control (Lewis et al., 1988), OsIQD14 was detected in the pellet fraction (Figure 4g), demonstrating that OsIQD14 directly binds MT.

To elucidate which part of the OsIQD14 protein localized to MT, we generated GFP fusion proteins consisting of only the N- or C-terminal region of OsIQD14. Although both MT and nuclear localization were observed in the p35S::OsIQD14-GFP line expressing the full-length protein in dividing cells (Figure S2d), the C-terminal region of OsIQD14 fused to GFP showed MT localization only, while the N-terminal region of OsIQD14 fused to GFP showed nuclear localization only (Figure S3a-3h), suggesting that in dividing cells, OsIQD14 could bind to some unknown protein by its N-terminus to mediate the nuclear localization. Taken together, these results show that OsIQD14 binds MT directly through its C-terminal domain.

In addition, we transformed both p35S::OsIQD14-C::GFP and p35S::OsIQD14-N::GFP into Arabidopsis and rice, but none of them showed difference compared to wild-type plants (pavement cell shape in Arabidopsis or seed size in rice). These results suggested that full-length IQD14 protein is necessary to affect grain size.

OsIQD14 controls cell shape by modulating microtubule behaviour

We wondered whether and how OsIQD14 could change the MT behaviour and dynamics. Based on the results described above and cortical MT localization of OsIQD14 in rice root cells and young leaves cells, it is also tempting to speculate that OsIQD14 controls spikelet hull cell shape by modifying the MT cytoskeleton. Unfortunately, the hardened spikelet hull cells in rice made observing MT ordering and dynamics technically impossible in our hands. Given that localization of the OsIQD14::GFP protein is conserved when expressed in Arabidopsis root and hypocotyl cells (Figure S2b), we used Arabidopsis as a heterologous system to analyse the dynamics and function of OsIQD14. Similar to AtIQD16 (Burstenbinder et al., 2017), Arabidopsis seedlings expressing p35S::OsIQD14::GFP showed narrow, long and spiralling cotyledons (Figure 5a,b). Also, epidermal pavement cells became largely isodiametric and lost the typical jigsaw puzzle shape found in control plants (Figure 5c, d). A similar effect on cell shape was observed for the spikelet hull cells themselves in rice (Figure 3j-i). These results indicate that we can indeed use Arabidopsis as a good model system to analyse OsIQD14 dynamics and function. Intriguingly, MT topology was strongly affected in p35S::OsIQD14::GFP plants compared to control plants as visualized by using p35S::GFP::TUA6 marker line (p35S::OsIQD14::GFP showed nuclear localization only, while the N-terminal region of OsIQD14 fused to GFP showed MT localization only), indicating that OsIQD14 also affects MT orientation. Moreover, hypocotyls of the dark-grown p35S::OsIQD14::GFP seedlings were highly sensitive to Oryzalin (Figure 5e-f), further supporting the role of OsIQD14 in controlling MT stability (Komaki et al., 2010; Nakamura et al., 2004). Further analysis of whether OsIQD14 overexpression could change the cell shape of Arabidopsis hypocotyl cells showed the much elongated hypocotyl cells of Arabidopsis lines expressing OsIQD14 (p35S::OsIQD14::GFP) compared to those of TUA6 marker line (p35S::GFP::TUA6, Figure S4c-e).
TUA6) into the p3SS::OsIQD14:GFP line. We next analysed MT dynamics in the presence or absence of OsIQD14 protein in dark-grown hypocotyl cells using spinning-disk confocal microscopy and observed that OsIQD14 overexpression strongly reduced MT dynamics (Figure S5a, b and Movies S1-S2). To further investigate how OsIQD14 could reduce the MT dynamics, we analysed time-lapse movies in more detail (Figure S5c-h). OsIQD14 overexpression caused more catastrophe events, with long time extension and more shrinking events (Figure S5d and Movies S3-S4), which we quantified by kymograph analysis (Figure S5e-i). Furthermore, co-localization of OsIQD14 with MTs showed punctate localization of OsIQD14 along MTs (Figure S5d and Movies S3 and S4), implying that OsIQD14 could interact with a microtubule-associated protein to change MT dynamics and orientation. Taken together, these results suggest that OsIQD14 reduces MT dynamics, which results in altered cell shapes.

Ca\textsuperscript{2+}-dependent binding of calmodulin to OsIQD14

As plants continuously adapt cell growth and expansion to ever-changing environmental conditions, multiple environmental (e.g. light) and developmental (e.g. hormones) inputs need to be translated into changes of the MT cytoskeleton. Considering the important function of OsIQD14 in controlling MT dynamics, we questioned how OsIQD14 activity itself could be controlled. Previously, interactions between IQD proteins and Calmodulins have been reported (Burstenbinder et al., 2017; Burstenbinder et al., 2013; Levy et al., 2005), but the biological significance of this binding remains unclear. Hence, we first tested the interaction between OsIQD14 and three rice Calmodulins, named OsCaM1, 2 and 3, all of which showed similar expression patterns to OsIQD14 in digital gene expression analysis (Figure S6a-c). Y2H and BiFC analyses showed that OsCaM1, 2 and 3 interact with OsIQD14 both in vitro (Figure 6a) and in vivo at MT structures in tobacco leaf epidermal cells (Figure 6b-g). Similar to other IQDs in Arabidopsis, OsIQD14 interaction with OsCaM1 was enhanced by calcium treatment (Figure 6h). We next aimed to investigate the biological significance of the OsCaM1-OsIQD14 interaction. Introducing a p3SS::OsCaM1 construct into the Arabidopsis p3SS::OsIQD14::GFP plants reverted all phenotypes related to OsIQD14 overexpression back to controls (Figure 5a-f). These included the long, narrow and spiralling cotyledons, and the absence of jigsaw-shaped epidermal pavement cells (Figure 5a-f). Taken together, these results suggest that the Ca\textsuperscript{2+}-dependent Calmodulin-OsIQD14 interaction inhibits OsIQD14

Figure 2 OsIQD14 regulates rice seed size. (a-f) Grain morphology of ZH11 and rice transgenic plants deficiency of (iqd14-C) or overexpressing OsIQD14 (p3SS::OsIQD14:GFP). Bar = 20 mm. (g-i) Measurement and statistical analysis of seed length and width (g), length/width ratio (h), and thousand-grain weight (i) of ZH11, iqd14-C and p3SS::OsIQD14::GFP plants. Data are shown as mean ± SE (n > 300). Statistical analysis was performed by calculating the P-value using a standard two-sided t-test; ***, P < 0.001; **, P < 0.01; *, P < 0.05. (j) Harvested panicles of one plant individual in ZH11, iqd14-C and p3SS::OsIQD14::GFP plants. Scale bar = 2 cm.
activity. Interestingly, the CaM-binding and MT-localization properties are separable: the CaM-interacting IQ67 domain is located in the N-terminus of OsIQD14, while the C-terminus is sufficient for MT localization (Figure S3g). Thus, the OsIQD14 protein likely recruits CaM to MT filaments through these two binding modules. Considering that multiple environmental (e.g. light) and developmental (e.g. hormones auxin) inputs induce transient increases in cytosolic Ca²⁺ (Babourina et al., 2002;
OsIQD14 protein is located at microtubules. (a-c) Subcellular localization of OsIQD14-GFP fusion protein in rice young root cells (a) or young leaves cells (b-c). Bar = 10 μm. (d-f) Subcellular localization of OsIQD14-GFP fusion protein in Arabidopsis hypocotyl cells under oryzalin treatment. Bar = 7 μm. (g) In vitro microtubule spin-down assay showed that OsIQD14 binds to microtubules directly. OsIQD14-His protein was incubated without or with taxol-stabilized bovine MT, divided into pellet (P) and soluble (Supernatant, S) fractions by ultracentrifugation, and then separated by 4%–12% SDS-PAGE and stained with Coomassie brilliant blue. MAP2 protein (280 kDa) that binds to microtubules is used as positive control.

Figure 4 OsIQD14 protein is located at microtubules. (a-c) Subcellular localization of OsIQD14-GFP fusion protein in rice young root cells (a) or young leaves cells (b-c). Bar = 10 μm. (d-f) Subcellular localization of OsIQD14-GFP fusion protein in Arabidopsis hypocotyl cells under oryzalin treatment. Bar = 7 μm. (g) In vitro microtubule spin-down assay showed that OsIQD14 binds to microtubules directly. OsIQD14-His protein was incubated without or with taxol-stabilized bovine MT, divided into pellet (P) and soluble (Supernatant, S) fractions by ultracentrifugation, and then separated by 4%–12% SDS-PAGE and stained with Coomassie brilliant blue. MAP2 protein (280 kDa) that binds to microtubules is used as positive control.
Monshausen et al., 2011), it is possible that this input will alter the activity of IQD protein by interacting with CaMs proteins, which in turn promotes MT dynamics to modify cell growth and expansion.

**Discussion**

Grain size and shape are key targets for rice improvement and invaluable agronomic traits. A range of grain size and/or shape regulators have been identified through studying natural variation or mutagenesis (Li and Li, 2016; Zheng et al., 2015). Identification of causal genes has revealed a clear role for transcription factors such as OsSPL16 (Wang et al., 2015), OsSPL13 (Si et al., 2016), OsGRF4 (Hu et al., 2015), a TRM-containing protein GW7 (Wang et al., 2015), an auxin-metabolism protein TGW6 (Ishimaru et al., 2013), and a plasma membrane-localized IQD protein GW5 (Duan et al., 2017; Liu et al., 2017), among others.

While these functions are diverse, no coherent mechanistic framework has emerged for the cellular basis underlying the altered morphology. Importantly, although GW5 and GW5 like (GW5L, Tian et al., 2018) share similarities with OsIQD14 regarding sequence and control of seed shape, both GW5 and GW5L are plasma membrane-localized protein involving in brassinosteroid signalling by regulating BIN2 activity. In contrast, we identified OsIQD14 as a key regulator of grain shape by acting on cell shape determination through controlling the MT cytoskeleton, making this a novel target for breeding approaches. In addition, GW5 and GW5L were shown to regulate the grain width by limiting cell proliferation in the spikelet hull through regulating BIN2 activity, while our studies showed that MT-binding OsIQD14 changes the cell shape of rice hull, suggesting that cell shape modification could also change the grains size, which is different from GW5 or GW5L. Intriguingly, while mutations and overexpression of OsIQD14 consistently affect grain properties, no adverse pleiotropic effects were observed. Thus, OsIQD14 allows direct modulation of MT behaviour and cell shape in husk cells to affect grain properties without the pleiotropic effects that manipulation of the MT cytoskeleton often induces (Hashimoto, 2015).

OsIQD14 is highly expressed in the rice panicle and SAM. However, when OsIQD14 was overexpressed in *Arabidopsis*, we did not find obvious difference in the inflorescence, flowers or

**Figure 5** OsIQD14 regulates cell shape of *Arabidopsis* by affecting microtubule ordering. (a-b) Seedling phenotype of wild type (Col-0) and *Arabidopsis* expressing p35S::OsIQD14::GFP (Bar = 0.5 cm). (c-d) Epidermal pavement cell phenotype of p35S::GFP:TUA6 in WT (Col-0) or *Arabidopsis* expressing p35S::OsIQD14::GFP (Bar = 50 μm). (e-f) Observation (e) and measurement (f) of hypocotyl lengths of dark-grown 4-day-old wild type (Col-0) and *Arabidopsis* seedlings expressing p35S::OsIQD14::GFP under oryzalin treatment. Data are shown as mean ± SE (n = 20). Statistical analysis was performed by calculating the P-value using a standard two-sided t-test; **, P < 0.01.
Figure 6 Calmodulins interact with OsIQD14. (a) Yeast-2-hybrid analysis showed that OsIQD14 interacts with OsCaM1, 2 and 3 directly in vitro. Full-length cDNAs of OsIQD14, OsCaM1, 2, 3 were subcloned into pGBKT7 or pGADT7 vectors. Transformed yeast cells were grown on synthetic dropout (SD-2, SD-Trp, SD-Leu; SD-3, SD-Trp, SD-His, SD-Leu) medium. Empty pGADT7 or pGBKT7 vectors transformed with OsIQD14-BD and OsCaM1/2/3-AD were used as negative controls. AD, activating domain; BD, binding domain. (b-g) BiFC assay showed that OsIQD14 interacts with OsCaM1 at microtubules in N. benthamiana epidermis cells by co-expression of OsIQD14-n/cYFP and OsCaM1-c/nYFP (Bar = 20 μm). (c) and (f) showed an enlarged view of interaction zone closed to plasma membrane (Bar = 5 μm). Empty n/c YFP was used as negative controls (d and g). (h) Calcium-dependent OsCaM1-OsIQD14 interaction in vitro. GST beads loaded with GST-tagged Calmodulin (OsCaM1) were incubated with OsIQD14-His protein at 4 °C in the presence of 2 mM CaCl₂ or 5 mM EGTA. Pellet (GST-OsCaM1 beads) fraction was detected by probing with His antibody (top) or stained with Coomassie brilliant blue (bottom).
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seeds. Considering the difference in plant architecture between monocots and dicots, this suggests that IQDs function differently in different plant species. Recent work on Arabidopsis IQDs showed that ectopic expression of AtIQD16 also leads to narrow, long and spiralling caryopses (Burstenbinder et al., 2017), suggesting that the basic function of IQDs in regulating MT dynamics is likely conserved.

Auxin has been suggested to influence MT dynamics, but the mechanism is largely unknown (Chen et al., 2014). Recent studies showed that auxin-mediated embryonic root formation may involve IQD proteins (Möller et al., 2017). OsIQD14 is the closest homolog of Arabidopsis AtIQD15-18 subclade, some of which are transcriptionally regulated by the AUXIN RESPONSE FACTORS/MONOPTEROS (ARF5/MP) transcription factor (Möller et al., 2017). The expression of OsIQD14 in rice was also induced by auxin, suggesting that rice and Arabidopsis IQDs may share a conserved upstream transcriptional regulatory mechanism. Experimental data from Arabidopsis provide evidence that auxin can mediate the microtubule reorganization within few minutes (Chen et al., 2014), and it will be interesting to test whether IQDs are involved in this rapid response. However, in our case, the hard hull cells of rice made it technically impossible to examine the microtubule dynamics. Using the Arabidopsis model to test the direct link between auxin/MTs and IQDs will expand our understanding of IQDs function on transferring signals to changes of the MT cytoskeleton. In addition, auxin can induce an increase of Ca2+ in few seconds (Monshausen et al., 2011), which could promote CaM binding to IQDs (Figure 6h). Intriguingly, we also found that OsIQD14 activity is regulated by CaMs (Figure S7). It is possible that high levels of Ca2+ will lead to the inhibition of IQD activity, which then regulates the behaviour of microtubules. Given that similar activities and regulation were found for the Arabidopsis orthologs of OsIQD14 (Burstenbinder et al., 2017), this module is likely deeply conserved in flowering plants.

Intriguingly, the phenotypes observed upon OsIQD14 overexpression in Arabidopsis seedlings are very similar to those observed in spiral2 mutants (Shoji et al., 2004; Wightman et al., 2013). SPIRAL2 (SPR2) is a plant-specific MT-binding protein required for anisotropic growth in Arabidopsis (Shoji et al., 2004; Wightman et al., 2013; Yao et al., 2008). Recent reports showed that SPR2 can protect MT minus ends to promote KATANIN-dependent severing and reorientation of plant cortical microtubule arrays (Nakamura et al., 2018). Our preliminary data from yeast two hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) analyses suggested that there is a direct interaction between OsIQD14 and SPR2 (Yang et al., 2018; Wendrich et al., 2018). However, it remains unclear how these proteins regulate each other. Based on the identical spiralling phenotype observed in IQD14 overexpression lines and the spr2 mutant, IQD14 might inhibit SPR2 activity. In spr2 mutants, the MT reorientation induced by a blue light stimulus was abolished (Nakamura et al., 2018). Notably, blue light also can trigger a calcium flux within seconds (Babourina et al., 2002). It is thus likely that CaMs, IQDs and SPR2 might form a multimeric protein complex, allowing to regulate each other’s activity. In this scenario, IQDs could inhibit SPR2 activity, which in turn could be blocked by calcium-mediated CaM binding to IQD14. Although it is tempting to propose a central role for IQD proteins in this process, a more detailed analysis of this putative protein complex will be required to fully understand this auxin-induced calcium flux and its link to microtubule reorientation in the coming years.

Experimental procedures

Plant materials and growth conditions

Rice (Oryza sativa, japonica variety Zhonghua11, ZH11) plants were cultivated in the field at Shanghai under natural growing conditions. For growth of transgenic plants, rice seeds were germinated in sterilized water and grown in a phytotron under a 12-h light (28 °C) cycle. Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used in all transformation and phenotype analysis. All seeds were germinated on MS (Murashige and Skoog, Duchefa) medium after three days at 4 °C. Seedlings and plants were grown in a phytotron at 22 °C with a 16-h light/8-h dark photoperiod.

Vector construction and plant transformation

The vector CRISPR/Cas9-iqd14 was generated by using two 20-bp fragments from the first exon of OsIQD14 introducing into pOsCas9 vector, and then, this plasmid was transformed into rice (ZH11) by Agrobacterium tumefaciens-mediated transformation (Hiei et al., 1994). The OsIQD14 CDNA was amplified by PCR with primers IQD14-1 and IQD14-2 (Table S1) using total cDNA of ZH11 seedlings as template and subcloned into pENTR/D-TOPO (Invitrogen) to generate the pENTR/D-TOPO-IQD14 construct. For stable transformation, p35S::OsIQD14::GFP was generated by LR reactions with pGW85 using pENTR/D-TOPO-IQD14, which was then transformed into ZH11. OsIQD14 expression levels of p35S::OsIQD14::GFP plants were examined by qRT-PCR, and confirmed positive lines were used for further analysis.

Transformation of Arabidopsis Col-0 plants was performed by the floral-dipping procedure. Plants expressing p35S::OsCaM1:RFP and p35S::OsIQD14::GFP were generated by introducing a p35S::OsCaM1::RFP and p35S::OsIQD14::GFP construct into p35S::OsIQD14:GFP plants. Primers are listed in Table S1.

Promoter–reporter gene fusion studies and GUS activity analysis

To analyse the expression pattern of OsIQD14 gene, a 700-bp DNA fragment of OsIQD14 promoter was ampliﬁed by PCR using ZH11 genomic DNA as template and subcloned into pENTR/D-TOPO vector. The resultant construct pOsIQD14::GUS was generated by LR reaction with pGW84 and transformed into ZH11, and confirmed positive lines were used for further analysis. GUS activity of T2 homozygous progeny of independent lines was detected according to previous description (Jefferson et al., 1987) and photographed using a Nikon SMZ 800 stereoscope with a Nikon digital Coolpix 995 camera.

RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Total RNAs were extracted using TRIzol reagent (Invitrogen) and reversely transcribed to first-strand cDNA. qRT-PCR analysis was performed with Real-Time PCR Master Mix (Toyobo), and data were collected using the Bio-Rad Real-Time Detection System in accordance with the manufacturer’s instruction manual. Primers were listed in Table S1. Expression of OsIQD14 was analysed using primers IQD14-RT1 and IQD14-RT2.

Scanning electron microscopy observation of spikelet hull

Number and area of cells at the outer parenchyma layer of the spikelet hulls were measured by Olympus stream software. The

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sample pretreatment for scanning electron microscopy observation (S-3000N; Hitachi) was performed as described previously (Liu et al., 2015). Grain weight was analysed as described previously (Fu and Xue, 2010).

Subcellular localization and cell shape analysis
Fluorescence of transgene seedlings and tobacco epidermal cells was observed by confocal laser scanning microscopy (Leica SP8) with an argon laser excitation wavelength of 488 nm or 561 nm. For imaging with SIM, the Alpha Plan Apochromat 100x, NA 1.57 oil objective was used, and images were acquired from a single optical section.

For spikelet hull cell morphology analysis, PI (10 μg/mL) was applied to rice young spikelet cells, which were then imaged by confocal.

MT dynamic analysis
Confocal imaging for MT dynamic analysis was performed with a Perkin-Elmer spinning-disk confocal with a 100X Plan-Apo 1.4 NA oil-immersion objective. GFP was excited at 488 nm, and RFP was excited at 561 nm. The time series used for comparing MT dynamics were acquired by exciting RFP:UAA6 with 400 ms exposures of 561 nm excitation at 20-s intervals over 30 min. Time-lapse imaging for the OsIQR4:GFP and RFP:UAA6 dual-labelled plants was performed at 20-s time intervals with 500 ms exposure. To measure the MT dynamics, we colour-coded the movies based on time (in which, for example, MT at frame 1 is red, MT at frame 6 green etc.). Hence, static MTs show as white structures (overlay of all colours: red, green, blue, cyan, magenta and yellow, respectively) and mobile MTs will be displayed as a multi-coloured line.

Protein–protein interaction assays
Interaction of OsIQR4 and OsCam1s was detected by standard Y2H analysis following the manufacturer’s instructions (Clontech). cDNAs encoding OsIQR4, OsCam1, 2, 3 were subcloned into pGBK7 and pGADT7 vector, resulting in the fusion vectors were co-expressed in BL21 DE3 cells, and expression of the fusion protein was induced by adding isopropyl-β-D-thiogalactoside (IPTG, final concentration 1 mM) and subcloned into pCold-HF for expression in BL21 DE3 at 30°C for 4 h by induction with 1 mM IPTG. Bacterial cells were harvested and sonicated in lysis buffer (50 mM Tris HCl, 150 mM NaCl). After centrifugation, the supernatant was used for incubating with GST agarose. Aliquots of 100 μL of OsCam1-GST beads, pre-equilibrated with lysis buffer, were mixed with 500 μL of bacterial supernatant supplemented with 2 mM CaCl2 or 5 mM EGTA and incubated for 1 h at 4°C under gentle shaking. OsCam1 beads were sedimented by centrifugation and washed four times with 500 μL of lysis buffer, followed by a final wash with 100 μL of the same solution. The bound proteins were eluted by boiling the beads for 2 min in 100 μL of 4× SDS sample buffer. Proteins of the total extract, the initial supernatant, the last wash, and the pellet fraction were analysed by SDS-PAGE and Western blot using antibody against His.

Expression of recombinant OsCam1 and calmodulin binding assay
Calmodulin binding assay was performed according to previous description with some modifications (Levy et al., 2005). For expression of OsCam1-GST, a full-length cDNA fragment encoding the OsCam1 was first amplified and then subcloned into pENTR/D-TOPO (Invitrogen). To express the OsCam1-GST fusion protein, pDEST-GST was used with Gateway LR Clonase II enzyme mix (Invitrogen). The recombinant OsCam1 protein was expressed in BL21(DE3) at 30°C for 4 h by induction with 1 mM IPTG. Bacterial cells were harvested and sonicated in lysis buffer (50 mM Tris HCL, 150 mM NaCl). After centrifugation, the supernatant was used for incubating with GST agarose. Aliquots of 100 μL of OsCam1-GST beads, pre-equilibrated with lysis buffer, were mixed with 500 μL of bacterial supernatant supplemented with 2 mM CaCl2 or 5 mM EGTA and incubated for 1 h at 4°C under gentle shaking. OsCam1 beads were sedimented by centrifugation and washed four times with 500 μL of lysis buffer, followed by a final wash with 100 μL of the same solution. The bound proteins were eluted by boiling the beads for 2 min in 100 μL of 4× SDS sample buffer. Proteins of the total extract, the initial supernatant, the last wash, and the pellet fraction were analysed by SDS-PAGE and Western blot using antibody against His.

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Conflicts of interests
The authors declare no conflicts of interests.

Author contributions
H.X. and D.W. conceived the project; B.Y. and J.R.W. designed the experiments; B.Y. performed the experiments; H.X., D.W. and B.D.R. supervised the project; and B.Y., B.D.R., D.W. and H.X. wrote the paper with input from all authors.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** In silico expression profile of *OsIQD14* and analysis of *OsIQD14* CRISPR/Cas9 lines.
**Figure S2** Subcellular localization of *OsIQD14* protein.
**Figure S3** Subcellular localizations of the N-terminus and C-terminus of *OsIQD14*.
**Figure S4** *OsIQD14* regulates cell shape of *Arabidopsis* by affecting microtubule ordering.
**Figure S5** *OsIQD14* affects MT behaviour.

**Figure S6** In silico expression profile of *OsCaM1*, 2 and 3 in rice various tissues.
**Figure S7** Pavement cell shape of *Arabidopsis* seedlings expressing *OsIQD14* and *OsCaM1*.

**Table S1** List of primers used in this study.

**Movie S1** Time series show the dynamic localization of RFP-TUA6 fusion protein (p35S::RFP:TUA6) in etiolated hypocotyl cells. Images were acquired at 20-s intervals.
**Movie S2** Time series show the dynamic localization of RFP-TUA6 fusion protein (p35S::RFP:TUA6) in etiolated p35S::OsIQD14::GFP hypocotyl cells. Images were acquired at 20-s intervals.
**Movie S3** Time series show the dynamic localization of OsIQD14-GFP fusion protein (green, p35S::OsIQD14::GFP) in etiolated hypocotyl cells. Images were acquired at 20-s intervals.
**Movie S4** Time series show the dynamic localization of OsIQD14-GFP fusion protein (green, p35S::OsIQD14::GFP) and RFP-TUA6-labelled cortical MTs (red, p35S::RFP:TUA6) in dark-grown hypocotyl cells. Images were acquired at 20-s intervals.