Research Article

Short title: OsRePRP-cytoskeleton interactions

Corresponding author: Tuan-Hua David Ho

An Intrinsically Disordered Protein Interacts with the Cytoskeleton for Adaptive Root Growth under Stress

An-Shan Hsiao\(a\), Kuan Wang\(b, c\) & Tuan-Hua David Ho\(a, d, 1\)

\(a\) Institute of Plant and Microbial Biology, Academia Sinica, Taipei, 11529, Taiwan
\(b\) Institute of Biological Chemistry, Academia Sinica, Taipei, 11529, Taiwan
\(c\) College of Biomedical Engineering, Taipei Medical University, Taipei 11031, Taiwan
\(d\) Biotechnology Center, National Chung Hsing University, Taichung 402, Taiwan

\(1\) Address correspondence to tho@gate.sinica.edu.tw

One-sentence summary: Rice REPETITIVE PROLINE-RICH PROTEIN affects cytoskeletal reorganization, cell wall remodeling and sucrose synthase activity as an adaptive response to water deficit in rice roots.

Author Contributions: A.-S.H. and T.-H.D.H. conceived the research plans; K.W. and T.-H.D.H. supervised the experiments; A.-S.H. performed the experiments; A.-S.H. designed the experiments and analyzed the data; A.-S.H. and T.-H.D.H. prepared the article; T.-H.D.H. agrees to serve as the author responsible for contact and ensures communication.

Funding information: This research was supported by grants (MOST 106-2311-B-001-020 and 108-2311-B-005-007) from Ministry of Science and Technology
and in part by the Advanced Plant Biotechnology Center from the Featured Area Research Center Program within the framework of Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan. A.-S.H. received a postdoctoral fellowship from MOST (MOST 106-2811-B-001-112).
Abstract

Intrinsically disordered proteins function as flexible stress modulators *in vivo* through largely unknown mechanisms. Here, we elucidated the mechanistic role of an intrinsically disordered protein, REPETITIVE PROLINE-RICH PROTEIN (RePRP), in regulating rice (*Oryza sativa*) root growth under water deficit. With nearly 40% proline, RePRP is induced by water deficit and abscisic acid (ABA) in the root elongation zone. RePRP is sufficient and necessary for repression of root development by water deficit or ABA. We showed that RePRP interacts with the highly ordered cytoskeleton components actin and tubulin, both *in vivo* and *in vitro*. Binding of RePRP reduces the abundance of actin filaments, thus diminishing non-cellulosic polysaccharide transport to the cell wall and increasing the enzyme activity of sucrose synthase. RePRP also reorients the microtubule network, which leads to disordered cellulose microfibril organization in the cell wall. The cell wall modification suppresses root cell elongation, thereby generating short roots, whereas increased sucrose synthase activity triggers starch accumulation in “heavy” roots. Intrinsically disordered proteins control cell elongation and carbon reserves via an order-by-disorder mechanism, regulating the highly ordered cytoskeleton for development of “short-but-heavy” roots as an adaptive response to water deficit in rice.
Introduction

Intrinsically disordered proteins lack a defined three-dimensional structure but often contain a simple amino acid composition with repeated sequences that provide the basis for multivalent intermolecular interactions (Uversky, 2016). With their unique structural flexibility, conformational adaptability and ability to react quickly in response to changing environments, intrinsically disordered proteins are often functional as hubs of protein–protein interaction networks in response to environmental stresses (Uversky, 2011). Recently, the crucial role of intrinsically disordered proteins in stress-triggered phase transition has been addressed (Ruff et al., 2018). As highlighted cases, tardigrade disordered proteins are essential for their high level of desiccation tolerance (Boothby et al., 2017), and disordered LATE EMBRYOGENESIS ABUNDANT (LEA) proteins often accumulate under dehydration and osmotic stresses in plants (Giarola et al., 2017).

Water deficit, caused by global climate changes and increasing world population, has been the most severe environmental stress to crop plants, affecting agricultural productivity and food security (Hu and Xiong, 2014). Water deficit triggers plants to produce abscisic acid (ABA), which orchestrates stress-specific responses and stress tolerance (Bray, 1997). The hormone ABA is also involved in seed dormancy and desiccation tolerance during seed development (Finkelstein et al., 2002). As a staple food feeding more than half of world populations, rice (Oryza sativa) requires two to three times more water than other dryland cereals do; hence improving the adaptation of rice to water-deficit conditions is critical (Kadam et al., 2017). Because the root is one of the organs perceiving water deficit, it is essential to understand the biological mechanism regulating rice root growth under water deficit (Rellán-Álvarez et al., 2016; Buckley, 2019).

As a physical barrier surrounding every plant cell, the cell wall is inherently involved
in regulating cell expansion (Bashline et al., 2014). The cell wall is composed of cellulose, non-cellulosic wall polysaccharide polymers such as hemicellulose and pectin, and a small amount of protein (Bashline et al., 2014). The complexity of the cell wall is partially controlled by the dynamic intracellular cytoskeleton (Szymanski and Cosgrove, 2009; Bashline et al., 2014). Microtubules guide the orientation of cellulose synthase complexes at the plasma membrane (Paredez et al., 2006; Crowell et al., 2009). Non-cellulosic polysaccharides are assembled within the Golgi, secreted and transported through Golgi-derived vesicles, and associated with newly synthesized cellulose microfibrils in the cell wall (Driouich et al., 1993). The transport of Golgi-derived vesicles containing hemicellulose and pectin is highly controlled by actin filaments (Baluska et al., 2002; Kim et al., 2005).

In animal cells, microcompartmentation of glycolytic enzymes with actin filaments generates metabolic channeling to favor the maximal reaction rates of carbohydrate metabolism (al-Habori, 1995; Real-Hohn et al., 2010). In plants, several enzymes involved in carbohydrate metabolism have been reported to interact with cytoskeleton proteins (Chuong et al., 2004; Balasubramanian et al., 2007; Garagounis et al., 2017). Among them, sucrose synthase has been shown to bind actin filaments both in vivo and in vitro (Winter et al., 1998; Duncan and Huber, 2007), but the functional significance of such binding has not been determined. Sucrose synthase catalyzes the reversible conversion of sucrose to/from NDP-glucose and fructose, playing an important role in conserving the energy in the nucleotide sugar products, which in turn are precursors for the synthesis of cellulose or starch (Winter and Huber, 2000; Koch, 2004). Sucrose synthase exists ubiquitously in plants and is particularly active in plant sink tissues, such as roots, developing seeds, young leaves or tubers (Winter and Huber, 2000; Koch, 2004).

Because of the versatility of intrinsically disordered proteins, they are relevant for
plant adaptation and survival under changing environments (Covarrubias et al., 2017).

A case in point is the LEA proteins, being involved in water deficit responses (Candat et al., 2014). However, because of technical and experimental barriers, research of intrinsically disordered proteins is limited in in vitro analyses, lacking in vivo mechanism and interaction evidence (Covarrubias et al., 2017). Proline-rich repeats/proteins often provide intrinsically disordered states and serve as environmental modulators (Reiersen and Rees, 2001). One superfamily of plant cell wall proteins, the hydroxyproline-rich glycoproteins (HRGPs), which are proline-rich and contain repeated sequence motifs and extensive glycosylation, have been implicated in many biological functions in plants (Johnson et al., 2017). Both proline-rich proteins and HRGPs are considered to be involved in the responses of plants to environmental factors (Sugimoto-Shirasu et al., 2004).

We have characterized an intrinsically disordered protein, Oryza sativa REPETITIVE PROLINE-RICH PROTEIN (OsRePRP), with extremely high proline content (nearly 40%), that is distinct from the classical HRGPs (Tseng et al., 2013). OsRePRP and its homologs are only identified in monocotyledonous plants that have an adventitious root system (Tseng et al., 2013), whereas most of the other proline-rich proteins with less proline content than OsRePRP and different structural motifs are found in the dicotyledonous plants with a tap root system (Johnson et al., 2017). All four genes (OsRePRP1.1, OsRePRP1.2, OsRePRP2.1 and OsRePRP2.2) in the OsRePRP family are upregulated in roots by ABA, salinity and water deficit (Tseng et al., 2013). OsRePRP2-overexpressing (OsRePRP2-OX) transgenic rice featured a short root and reduced cell length phenotype, resembling the repressed root growth with water deficit or ABA in wild-type rice (Tseng et al., 2013). Conversely, knockdown of both OsRePRP1.1 and OsRePRP2.1 in OsRePRP RNA-interference
(OsRePRP-Ri) transgenic rice reduced the repression effect on root growth, which indicates that OsRePRPs play a necessary and sufficient role in water deficit- or ABA-regulated root development (Tseng et al., 2013).

Here we report that OsRePRPs interact with the cytoskeleton components actin and tubulin specifically, which leads to a major re-orientation of cellulose microfibrils in the cell wall to inhibit the elongation of root cells but promote more starch accumulation in the “short-but-heavy” roots. Our results demonstrate a novel role of an ABA/stress-induced intrinsically disordered protein in regulating plant root growth under water deficit.

Results

Recombinant OsRePRPs Bind to Actin and Tubulin in Vitro

OsRePRP protein sequences are rich in the amino acid (aa) proline, nearly 40%. When OsRePRP sequences were assessed by using four different Predictor Of Natural Disordered Regions (PONDR) predictors, VSL2, VL3, VL-XT and XL1-XT (Xue et al., 2010), both OsRePRP1.1 and OsRePRP2.1 showed high disorder scores (> 0.5) among the major protein sequences (Supplemental Figure S1A and S1B). The internal duplication pattern was also observed in the repetitive regions of both OsRePRP1.1 and OsRePRP2.1 (Supplemental Figure S1C and S1D). Segmental repeat organization of OsRePRPs includes main repeat regions and non-repeat regions near the termini (Supplemental Figure S2). Using RADAR software, we dissected the repeat regions of OsRePRP1.1 and OsRePRP2.1 into several super-repeat segments (Figure 1A; Supplemental Figure S3A). In OsRePRP1.1, the segments are mainly composed of PEPK repeats, whereas in OsRePRP2.1, they are PQPN/PDPK repeats (Figure 1A; Supplemental Figure S3A). The PEPK motifs are very similar to the actin-binding PEVK repeats in animal titin, which controls the passive elasticity of the sarcomere in...
striated muscle cells (Gutierrez-Cruz et al., 2001). The architecture composed of single repeats, super repeats and non-repeat regions bears a remarkable resemblance to nebulin (Wang et al., 1996), a muscle actin thin filament “ruler.” Therefore, we expressed recombinant OsRePRPs (rOsRePRPs) and their serial segments in *Pichia pastoris* and *Escherichia coli* to test the binding of actin and tubulin by using microscale thermophoresis (MST) technology (Wienken et al., 2010) and determined the equilibrium dissociation constant (*K*<sub>d</sub>).

MST assays showed rOsRePRP1.1 and rOsRePRP2.1 as well as their serial segmental recombinant proteins/peptides bound to rabbit muscle actin and porcine brain tubulin with various *K*<sub>d</sub> (Figure 1B; Supplemental Figure S3B). Recombinant OsRePRP2.1 bound to actin with *K*<sub>d</sub> 1.41 μM and to tubulin with *K*<sub>d</sub> 4.32 μM (Figure 1B), whereas rOsRePRP1.1 bound to actin with *K*<sub>d</sub> 1.72 μM and to tubulin with *K*<sub>d</sub> 6.54 μM (Supplemental Figure S3B). The *K*<sub>d</sub> of serial segmental rOsRePRP2.1 binding to actin/tubulin ranged from 0.174 to 4.56 μM and the synthetic one-super-repeat peptides of OsRePRP2.1 bound to actin and tubulin from 0.763 to 1.71 μM (asterisks in Figure 1B). The *K*<sub>d</sub> of serial segmental rOsRePRP1.1 binding to actin/tubulin ranged from 0.296 to 24.8 μM (Supplemental Figure S3B). Consistent with our previous report (Tseng et al., 2013), rOsRePRP1.1 and rOsRePRP2.1 interacted with arabinogalactan (AG) at *K*<sub>d</sub> 52.1 and 74 μM, respectively, but not with sucrose (Supplemental Figure S3C and S3D). As control experiments, no binding was observed between the negative bovine serum albumin (BSA) control and rOsRePRP1.1 or rOsRePRP2.1 (Supplemental Figure S3E and S3F). We also tested two other recombinant plant proteins: recombinant RICE BIG GRAIN1 (rOsRBG1) and one intrinsically disordered LEA protein from barley, rHvHAV1. Neither showed binding to actin/tubulin (Supplemental Figure S3G and S3H). The results obtained from the control experiments suggested that the binding of OsRePRPs to actin and...
tubulin is not due to non-specific interactions.

We used competition binding assays to verify segmental rOsRePRP2.1\textsuperscript{60-179} binding to AG, actin and tubulin (Figure 1C and 1D). Recombinant OsRePRP2.1\textsuperscript{60-179} was first mixed with AG for measuring the binding of rOsRePRP2.1\textsuperscript{60-179} to actin and tubulin. In the presence of 250 \(\mu\)M AG, rOsRePRP2.1\textsuperscript{60-179} retained the binding affinity to actin and tubulin, with \(K_d\) 0.663 and 0.509 \(\mu\)M, respectively (Figure 1C and 1D pink-dashed curves). Thus, the binding of rOsRePRP2.1\textsuperscript{60-179} to actin and tubulin is independent of its binding to AG \textit{in vitro}. To further verify the correlation between actin and tubulin binding, rOsRePRP2.1\textsuperscript{60-179} was first mixed with tubulin for measuring the binding of rOsRePRP2.1\textsuperscript{60-179} to actin and vice versa (Figure 1C and 1D). In the presence of 25 \(\mu\)M tubulin, the binding of rOsRePRP2.1\textsuperscript{60-179} to actin was greatly reduced (Figure 1C closed squares), and in the presence of 57.5 \(\mu\)M actin, the binding of rOsRePRP2.1\textsuperscript{60-179} to tubulin was barely detectable (Figure 1D closed squares). Therefore, actin and tubulin competed with each other for binding to rOsRePRP2.1\textsuperscript{60-179}. Similar competition between actin and tubulin was observed in the binding of the one-super-repeat peptide OsRePRP2.1\textsuperscript{60-87}, and AG did not seem to affect the peptide OsRePRP2.1\textsuperscript{60-87} binding to actin or tubulin much (Figure 1E and 1F).

To confirm the binding of rOsRePRP to actin filaments (F-actin) and microtubules, we used high-speed co-sedimentation assays with mixtures of rOsRePRP2.1\textsuperscript{60-179} and F-actin and microtubules polymerized to a steady state (Supplemental Figure S3I and S3J). High-speed centrifugation separated F-actin and microtubules in the pellet from G-actin and \(\alpha\beta\)-tubulin dimers in the supernatant. Besides binding to G-actin and \(\alpha\beta\)-tubulin dimers (Figure 1B to 1D), rOsRePRP2.1\textsuperscript{60-179} also bound to F-actin and microtubules (Supplemental Figure S3I and S3J).
Co-localization of OsRePRP2 and Cytoskeleton Markers in Rice Protoplasts

The MST binding assays suggested that OsRePRPs interact with the cytoskeleton proteins actin and tubulin *in vitro* (Figures 1 and Supplemental Figure S3), so we further investigated whether OsRePRP2 interacts with the cytoskeleton proteins *in vivo* by using the rice root protoplast transient-expression system. LifeAct has been reported to bind eukaryotic actin filaments (Riedl et al., 2008) and to be useful for studying the actin cytoskeleton in a wide range of plant lineages (Era et al., 2009). A microtubule reporter gene created by fusing the microtubule binding domain (MBD) of the mammalian microtubule-associated protein 4 (MAP4) gene with the green fluorescent protein (GFP) gene could be used to visualize microtubule orientation in plants (Marc et al., 1998). Hence, we constructed the cytoskeleton marker LifeAct fused with C-terminal GFP (LifeAct-GFP) and MBD fused with N-terminal GFP (GFP-MBD) to verify the subcellular co-localization with OsRePRP2 fused with C-terminal DsRed (OsRePRP2-DsRed) in the rice root protoplast transient-expression system (Figure 2). The results suggested that OsRePRP2-DsRed showed similar filamentous expression patterns and co-localized with both LifeAct-GFP and GFP-MBD in the rice root protoplasts (Figure 2A to 2H). The negative control DsRed was distributed throughout the cytosol and did not show similar filamentous patterns as LifeAct-GFP or GFP-MBD (Figure 2I to 2P). As control experiments, protoplasts expressing LifeAct-GFP and GFP-MBD did not show any fluorescence signals in the DsRed channel (Figure 2Q to 2T), excluding the possibility of channel bleeding. Pearson correlation coefficient analysis was used to quantify the co-localization (Figure 2U): OsRePRP2-DsRed co-localized with LifeAct-GFP (R = 0.81) and with GFP-MBD (R = 0.85) (Figure 2C, 2G and 2U), which was distinct from the negative control DsRed correlated with LifeAct-GFP (R = 0.41) and with GFP-MBD (R = 0.33) (Figure 2K, 2O and 2U).
OsRePRP Interacts with Actin and Tubulin in Vivo

To further investigate whether OsRePRP forms complexes with actin and tubulin in planta, GFP, OsRePRP1-GFP and OsRePRP2-GFP transgenic rice root cells were examined in co-immunoprecipitation (co-IP) assays with anti-GFP antibodies immobilized on magnetic beads. Actin and tubulin co-immunoprecipitated with anti-GFP beads in OsRePRP1-GFP and OsRePRP2-GFP but not GFP root extracts (Supplemental Figure S4A and S4B). On LC-MS/MS, the detection of actin and tubulin peptides further supported that OsRePRPs may interact with actin and tubulin in planta (Supplemental Tables S1 and S2). The in vivo interaction of OsRePRP-GFP and actin and tubulin was further confirmed by immunogold-labeling transmission electron microscopy (TEM), showing OsRePRP-GFP immuno-labelled by 18-nm gold particles and actin and tubulin by 12-nm gold particles (Figure 3A-3I). In practice, 5-15 nm gold particles are situated from the epitope at a distance of about 30 nm (Bergersen et al., 2008), so the distance < 60 nm between two sizes of gold particles was assumed to be co-localization. Most of the closest distances between OsRePRP-GFP and actin/tubulin were < 40 nm (Figure 3C-3D and 3H-3I), but most of the closest distances between GFP and actin/tubulin were > 100 nm (Supplemental Figure 4C-4F). These results also suggest that OsRePRPs interact with both types of cytoskeleton filaments in vivo.

Overexpression of OsRePRP2 Causes Reduced Abundance of Actin Filament

OsRePRP2 expression was highly induced by ABA (Supplemental Figure S5), and OsRePRP2-OX transgenic rice featured a short-root and reduced cell-length phenotype (Tseng et al., 2013; Supplemental Figure S6A), resembling the repression of root growth with water deficit or ABA in wild-type rice. This phenotype was
reduced in OsRePRP-Ri transgenic rice roots (Supplemental Figure S6A), which suggests that OsRePRP is necessary and sufficient in stress/ABA repression of root elongation.

Cytoskeleton filaments play an important role in the spatial control of cell expansion (Smith and Oppenheimer, 2005). Because OsRePRPs bind to actin and tubulin in vitro (Figure 1; Supplemental Figure S3), we wondered whether the short root and reduced cell length phenotype of OsRePRP2-OX transgenic rice was related to cytoskeleton filaments. Hence, we examined the in vivo actin filament (F-actin) organization of wild-type, OsRePRP2-OX and OsRePRP-Ri transgenic rice roots by Alexa Fluor 488-phalloidin staining.

Phalloidin specifically binds to F-actin (Wulf et al., 1979), so the fluorescence intensity on staining is positively correlated with the abundance of F-actin within cells. The overall fluorescence signal of F-actin in elongation-zone cells was weaker in OsRePRP2-OX than wild-type or OsRePRP-Ri roots (Figure 4A to 4C, Supplemental Video). The mean fluorescence intensity of F-actin was reduced by nearly five-fold for OsRePRP2-OX, with no significant difference between wild-type and OsRePRP-Ri roots (Figure 4G). Under ABA treatment, F-actin level was decreased in the wild type and barely visible in OsRePRP2-OX, but this phenomenon was less apparent in OsRePRP-Ri roots (Figure 4D to 4F, Supplemental Video). The mean F-actin fluorescence intensity was about three-fold higher in OsRePRP-Ri than wild-type roots (Figure 4G). The same patterns were also observed in the root division and differentiation zones (Supplemental Figure S7) and OsRePRP showed similar phenotypic changes with PEG stress treatment as with ABA treatment (Supplemental Figure S8). Thus, water deficit- or ABA-induced OsRePRP may be involved in regulating F-actin level primarily in the root elongation zone, but also in division and differentiation zones.
The root protoplasts of the wild type, OsRePRP2-OX and OsRePRP-Ri were isolated and transfected with LifeAct-GFP with the DsRed transfection control (Figure 5). In 5-day-old protoplasts, LifeAct-GFP in both wild-type and OsRePRP-Ri protoplasts showed filamentous patterns (Figure 5A and 5C), whereas LifeAct-GFP in OsRePRP2-OX protoplasts mainly showed cytosolic patterns but few filamentous patterns (Figure 5B). These results exclude the possibility that reducing F-actin level in OsRePRP2-OX was an artefact caused by the differential staining of phalloidin.

Microtubule Orientation is Altered in OsRePRP2-OX

We also examined microtubule organization of wild-type, OsRePRP2-OX and OsRePRP-Ri rice root cells under control and ABA treatment by immunofluorescence staining with anti-α-tubulin antibodies (Figure 6A to 6F). The microtubule network was less organized in OsRePRP2-OX than wild-type or OsRePRP-Ri root cells (Figure 6A to 6C). With ABA treatment, the disordered microtubule phenotype was apparent in the wild type but less so in OsRePRP-Ri cells (Figure 6D and 6F).

Further study in the rice root protoplast transient-expression system indicated that GFP-MBD showed a similar filamentous expression pattern in the wild type, OsRePRP2-OX and RePRP-Ri (Figure 6G to 6L). The microtubule organization in the rice root protoplast transient-expression system did not show the transverse arrangement shown in the whole-mount immunostaining of rice roots (Figure 6A). The results suggested that without the cell wall, the microtubule orientation of OsRePRP2-OX did not differ substantially from the wild type and RePRP-Ri (Figure 6G to 6I).

OsRePRP Affects Trafficking of Non-cellulosic Polysaccharides and Cell Wall

Cellulose Microfibril Organization
The cytoskeleton is involved in plant cell shape determination by affecting the patterns by which cell wall materials are deposited (Smith and Oppenheimer, 2005). F-actin plays a role in vesicle trafficking of non-cellulosic polysaccharides (Baluska et al., 2002; Kim et al., 2005). Microtubules maintain the cellulose synthase complexes localized at the plasma membrane to guide cellulose deposition on the cell wall (Paredez et al., 2006; Crowell et al., 2009). Because OsRePRP regulates actin filament distribution and microtubule organization in vivo, we wondered whether OsRePRP affects non-cellulosic polysaccharide secretion on the cell wall and cell-wall cellulose microfibril arrangement. By using metabolic click-labeling with fucose alkyne (FucAl) and Alexa Fluor 488-azide (Anderson et al., 2012), we monitored FucAl incorporation into the cell wall by confocal microscopy (Figure 7A to 7C). FucAl-associated fluorescence was observed in the wild-type and OsRePRP-Ri cell wall, with less fluorescence observed in the OsRePRP2-OX cell wall (Figure 7A to 7C). The quantitative data indicate that the fluorescence signal in OsRePRP2-OX was reduced about 15-fold as compared with the wild type or OsRePRP-Ri (Figure 7D). Hence, the metabolic process of FucAl incorporation in the cell wall may be blocked in OsRePRP2-OX. Field emission scanning electron microscopy of cells in the root elongation zone revealed a transverse cell-wall cellulose microfibril network in the wild type (Figure 7E), which agrees with the previous findings of a transverse cellulose microfibril-aligned pattern perpendicular to the direction of cell elongation (Sugimoto et al., 2000). OsRePRP2-OX showed a more disorganized orientation of cellulose fibril network (Figure 7F) as compared with the transverse pattern of wild-type and OsRePRP-Ri networks (Figure 7E and 7G). Under ABA treatment, the transversely ordered cellulose fibril network of the wild type became disordered (Figure 7H), with some thick fibrils in a disarranged cellulose network observed in OsRePRP2-OX.
(Figure 7I), whereas OsRePRP-Ri still retained some of the transversely ordered cellulose fibril network (Figure 7J). The quantitative directionality (orientation in terms of angle) analysis has been used in a study of collagen fibril alignment (Grossman et al., 2016) and we applied herein in the quantitative estimation of the orientation of cell wall cellulose microfibrils. The quantitative directionality analyses showed that cellulose microfibrils of both the wild type and OsRePRP-Ri were aligned in one dominant direction (nearly 0° of orientation), whereas those of OsRePRP2-OX were dispersed and oriented in a multitude of directions (Figure 7K). After ABA treatment, the wild type and OsRePRP2-OX showed dispersed directions, but OsRePRP-Ri retained the dominant direction alignment (nearly 0° of orientation) (Figure 7L). Hence, OsRePRPs may affect root cell wall cellulose microfibril formation in vivo.

**Starch Accumulation in “Heavy” Roots under PEG Stress**

Besides a short root phenotype (Supplemental Figure 6A), OsRePRP2-OX also displayed a “heavy” root phenotype (Figure 7M and 7N). In distilled water, root-tip segments of wild-type and OsRePRP-Ri plants floated on the water surface, whereas OsRePRP2-OX segments submerged beneath the surface of water, which suggests that OsRePRP2-OX segments were “heavy” (Figure 7M). After ABA treatment, wild-type root-tip segments also submerged, resembling the “heavy” root phenotype of OsRePRP2-OX under the control condition (Figure 7M). However, after ABA treatment, the “heavy” root phenotype was less apparent in OsRePRP-Ri than the wild type (Figure 7M). The quantification of dry weight per root length per seedling also confirmed the “heavy” root phenotype (Figure 7N).

We wondered whether storage starch accumulation contributed the biomass accumulation in OsRePRP2-OX, so we used iodine staining to address this question.
Rice roots accumulated starch under PEG stress, as shown by the dark iodine-stained color (Figure 8A and 8B). OsRePRP2-OX showed more dark-stained roots than the wild type or OsRePRP-Ri did after PEG treatment (Figure 8B). Sectioning was conducted to further observe the starch accumulation phenotype (Figure 8C to 8Q; Supplemental Figure S9). Under control conditions, some starch granules were observed in endodermal cells of OsRePRP2-OX (Figure 8D, 8G and 8J; Supplemental Figure 9B) but not in the wild type or OsRePRP-Ri (Figure 8C, 8E, 8F, 8H, 8I and 8K; Supplemental Figure S9A and S9C). No starch granules were observed in epidermal cells in the wild type, OsRePRP2-OX or OsRePRP-Ri (Supplemental Figure S9D to S9F). Under PEG stress, starch granules were accumulated in endodermal and epidermal cells in the wild type, OsRePRP2-OX and OsRePRP-Ri (Figure 8L to 8Q; Supplemental Figure S9G to S9I). OsRePRP2-OX showed the most severe starch accumulation phenotype (Figure 8P; Supplemental Figure S9H), with not much difference between the wild type and OsRePRP-Ri (Figure 8O and 8Q; Supplemental Figure S9G and S9I). In starch-accumulated cells, some very huge starch granules were observed in OsRePRP2-OX (Supplemental Figure S9H) but not in the wild type or OsRePRP-Ri, showing small and uniformed-sized starch granules (Supplemental Figure S9G and S9I). Starch content measurements also showed high starch accumulation in OsRePRP2-OX (Figure 8R), which suggests that OsRePRPs may play a role in storage nutrient accumulation under PEG stress.

Sucrose Synthase Enzyme Activity Increased in OsRePRP2-OX

Sucrose synthase is a key enzyme in cleaving sucrose to provide NDP-glucose for starch biosynthesis *in vivo* (Koch, 2004). From the previous co-IP experiments using anti-GFP antibodies, peptides of sucrose synthase were detected in OsRePRP1-GFP and OsRePRP2-GFP but not GFP transgenic plants by LC-MS/MS analysis.
(Supplemental Table 3). The co-immunoprecipitation of sucrose synthase in OsRePRP-GFP transgenic plants was also confirmed by western blot analysis (Figure 9A), most likely due to sucrose synthase binding to actin filaments in vivo (Winter et al., 1998). We wondered whether OsRePRP can affect the enzyme activity of sucrose synthase by reducing the amount of actin filaments. Thus, we examined the enzyme activity of sucrose synthase in both cleavage and synthetic directions of the wild type, OsRePRP2-OX and OsRePRP-Ri (Figure 9B and 9C). The sucrose synthase activity in the cleavage direction was two-fold higher in OsRePRP2-OX than the wild type or OsRePRP-Ri, whereas the activity in the synthetic direction was lower in OsRePRP2-OX than the wild type or OsRePRP-Ri (Figure 9B and 9C). Because the enzyme activity of sucrose synthase in maize was associated with its phosphorylation (Huber et al., 1996), we examined the phosphorylation status of sucrose synthase in the wild type, OsRePRP2-OX and OsRePRP-Ri by using PhosTag western blot analysis (Figure 9D). Both non-phosphorylated and phosphorylated forms of sucrose synthase were detected in the wild type, OsRePRP2-OX and OsRePRP-Ri, with no differences in levels (Figure 9D), which suggests that the phosphorylation status of sucrose synthase was not affected by OsRePRP. Thus, OsRePRP may affect sucrose synthase enzyme activity by interacting with actin filaments but does not affect its phosphorylation in planta.

Discussion

Herein, we presented a novel case that an intrinsically disordered protein specially interacts with the cytoskeleton proteins actin and tubulin to regulate development and biochemistry in rice root cells under water deficit. The studies of OsRePRP not only expand our knowledge of the roles and diversity of plant intrinsically disordered proteins but also foster communication across fields of plant and animal biology.
OsRePRPs are composed of PEPK repeats in OsRePRP1 and PQPN/PDPK repeats in OsRePRP2 (Figure 1A; Supplemental Figure S3A). OsRePRP1.1 and OsRePRP1.2 share 84% sequence identity and OsRePRP2.1 and OsRePRP2.2 share 94% sequence identity (Tseng et al., 2013). OsRePRPs are highly induced by ABA and water deficit, as shown by in situ hybridization studies and transcriptional analysis (Tseng et al., 2013; Supplemental Figures S5 and S10). The induction of OsRePRP expression by ABA was highly repressed in the double-gene knockdown line OsRePRP-Ri as compared with the wild type (Supplemental Figure S10). Thus, OsRePRP1 and OsRePRP2 may share similar functions in ABA/water deficit repressing root development. However, we did not observe any obvious phenotypic changes of OsRePRP1-OX as compared with the wild type (Supplemental Figure S6B), and only the double-gene knockdown line OsRePRP-Ri but not the single-gene knockdown lines OsRePRP1-Ri or OsRePRP2-Ri showed the reduced ABA effect (Tseng et al., 2013; Supplemental Figure S6A). Thus, we cannot rule out that OsRePRP1 may also play a similar role as OsRePRP2 and conclude that OsRePRPs are sufficient and necessary for ABA/water deficit repression of root development.

In the face of adversity and danger, animals can escape by using highly modulated skeletons and muscles. Titin and nebulin rule myosin and actin contraction in muscle cells, controlling muscle contraction (Labeit and Kolmerer, 1995; Wang et al., 1996; Gutierrez-Cruz et al., 2001; Ma and Wang, 2002). PEVK motifs of titin are repetitive and intrinsically disordered, with highly charged residues (Labeit and Kolmerer, 1995; Gutierrez-Cruz et al., 2001). Tandem repeats of titin and nebulin provide the binding sites for actin and myosin and scaffolding/crosslinking to the filamentous structures (Labeit and Kolmerer, 1995; Wang et al., 1996; Gutierrez-Cruz et al., 2001; Ma and Wang, 2002). OsRePRPs also showed repetitive (PEPK, PQPN and PDPK), intrinsically disordered and internal duplication patterns (Figure 1A; Supplemental...
Figures S1, S2 and S3) similar to titin and nebulin. Although rooted plants are sessile and do not have muscle cells to escape from environmental stresses, disordered OsRePRPs can function to regulate highly organized cytoskeleton filaments (actin and tubulin) to adapt plant growth under stress (Figures 4 to 6; Supplemental Figures S6A, S7 and S8). Similar to titin and nebulin controlling the elasticity of mammal muscle cells (Ma and Wang, 2002), OsRePRP regulates the adjustable elasticity needed for cell expansion in rice by directly binding to actin and tubulin (Figure 1; Supplemental Figure S3). Other than the studies on the cytoskeleton reorganization in response to abiotic and biotic stresses (Wang et al., 2011; Fujita et al., 2013; Li et al., 2015), OsRePRP is a novel case of an intrinsically disordered protein regulating the cytoskeleton in plants. Our finding suggests a regulation of highly ordered filamentous structures by repetitive disordered proteins in both plants and animals.

Our previous study mainly emphasized the plasma membrane localization of OsRePRPs (Tseng et al., 2013). However, the cytosolic localization of OsRePRPs in both cell fractionation and transient-expression experiments was also evident (Tseng et al., 2013). Since OsRePRPs contain signal peptides (Tseng et al., 2013), two possibilities may explain their localizations. First, signal peptides may act co-translationally or post-translationally (Panzner et al., 1995), leading to the presence of a protein in both the cytosol and the plasma membrane. Second, the primary amino acid sequence prediction may not precisely reflect the protein localization affected by protein-protein interactions (Koroleva et al., 2005). The immunogold-TEM observations presented here (Figure 3 and Supplemental Figure S4G) and our previous publication (Tseng et al., 2013) both support the notion that OsRePRPs appear to be present in both the plasma membrane and the cytosol.

In the rice root protoplast transient-expression system, OsRePRP2-DsRed was primarily expressed in the cytosol with some punctuated patterns (Supplemental
Figure 11). While overexpressing other cytoskeleton-binding proteins LifeAct-GFP and GFP-MBD, OsRePRP2-DsRed showed more obvious filamentous patterns co-localized with LifeAct-GFP and GFP-MBD (Figure 2). These results suggested that intrinsically disordered OsRePRP may have multifaceted functions affected by other cytoskeleton-binding proteins and various environmental conditions, thus OsRePRP2-DsRed showed distinct co-localized patterns with both LifeAct-GFP and GFP-MBD. Nevertheless, our co-IP dataset did not reveal other cytoskeleton-binding proteins that may interact with OsRePRPs. The rice genome may have not been fully annotated, and more studies regarding this issue should be conducted in the future.

The coupling between the cytoskeleton and extracellular matrix (ECM) in animals is well understood by the study of integrin-adhesion-receptor signaling (Schwartz and Ginsberg, 2002). However, in higher plants, critical linker molecules between the cytoskeleton and ECM (the cell wall in plants) are still missing (Baluska et al., 2003).

With reduced F-actin amount and disoriented microtubule organization (Figures 4 to 6; Supplemental Figure S7), OsRePRP2-OX transgenic rice showed impaired cell-wall polysaccharide deposition and disordered cellulose microfibril organization (Figure 7A to 7L). Thus, OsRePRP may affect non-cellulosic polysaccharide trafficking and cellulose patterning by altering the F-actin network and microtubule arrangement. From our results, we propose that OsRePRP is a stress-induced cytoskeleton–cell wall modulator. Unlike the transmembrane receptor integrins in animals (Schwartz and Ginsberg, 2002), OsRePRP has a distinct role in regulating the very dynamic interactions between the cytoskeleton and cell wall in plants.

Although sucrose synthase interacting with actin filaments in vivo is assumed to be a control mechanism (Koch, 2004), we lack direct evidence to support this idea. Herein, we showed increased sucrose synthase enzyme activity in OsRePRP2-OX (Figure 9B), so reducing F-actin may affect the enzyme activity of sucrose synthase in vivo.
Phosphorylation of sucrose synthase in maize has been reported to be associated with its enzyme activity (Huber et al., 1996). However, in our case, the increase in sucrose synthase enzyme activity was not associated with its phosphorylation (Figure 9D), distinct from the regulation explored in maize sucrose synthase (Huber et al., 1996).

In animals, cytoskeleton remodeling releases more free forms of aldolase, thereby increasing the glycolysis flux (Hu et al., 2016). Reducing F-actin level in OsRePRP2-OX may increase sucrose synthase activity by releasing more free forms of sucrose synthase, similar to the case in animal aldolase (Hu et al., 2016). However, whether OsRePRP interacts with sucrose synthase directly or indirectly (through actin filaments) remains to be investigated. However, our observations have to be interpreted with caution because the cortex cells in the cross sections of WT, OsRePRP2-OX and OsRePRP2-Ri look quite different in morphology (Fig 8) probably due to the effect of ReOsPRP2 on root elongation. Although the samples were sectioned at the same distance from root tips, i.e., 0.5 cm. they may reflect different root developmental stages caused by the differential expression of OsRePRP2. Nevertheless, the differences of SUS activities can at least partially account for the different level of starch accumulations in these samples. The interaction (direct or indirect) of OsRePRPs with sucrose synthase may not be only related to starch biosynthesis but also to cell wall metabolism by providing NDP-glucose as the substrate.

Our current study explains the reduced cell length phenotype of OsRePRP2-OX in our previous study (Tseng et al., 2013) and supports that both microtubules and actin filaments are critical for cell expansion (Smith, 2003). Observing disorganization of cell wall cellulose microfibrils and cortical microtubules in OsRePRP2-OX (Figures 6 and 7), people may expect to see a phenotype of anisotropic cell expansion rather than just reduced cell elongation. However, previous studies have shown that the degree of
growth anisotropy was not correlated with the degree of alignment of either microtubules or microfibrils (Baskin et al., 1999) and cell expansion in longitudinal and radial directions can be regulated independently in roots (Liang et al., 1997; Baskin, 2005). Hence, the phenotype of OsRePRP2-OX may probably support the notion that anisotropic cell expansion and longitudinal cell elongation are regulated independently in rice roots. The ultimate goal of ABA/water deficit-induced OsRePRP regulation on cytoskeleton dynamics and cell wall organization is to aid plant survival under adversity. Because ABA plays a role in seed dormancy and desiccation tolerance during seed maturation, the function of ABA-inducible OsRePRP is analogous to the action of ABA in seeds to halt root elongation and accumulate more starch (Figure 8; Supplemental Figures S6A and S9), thus shifting the roots to a dormant storage organ resembling seeds.

This “short-but-heavy root” strategy is similar to the rice flooding-tolerance gene SUBMERGENCE-1, which causes growth quiescence during flooding that is associated with the capacity for regrowth on desubmergence (Fukao et al., 2006; Xu et al., 2006). Thus, the “quiescence” concept may help plants overcome water deficit and water stresses. Highly charged intrinsically disordered proteins have high hydration capacities and preference for absorption of charged solute ions such as dehydrins to protect plants against water loss (Tompa et al., 2006; Uversky, 2011). Of note, disordered OsRePRP also functions in the water deficit response.

Overall, our study highlights the importance of OsRePRP in cytoskeleton–cell wall regulation and cytoskeleton-mediated cellular processes and suggests a common “order by disorder” mechanism in both plants and animals as well as a practical basis for crop improvement designs.

Materials and Methods
Protein Sequence Analysis

OsRePRP sequences were assessed by using four different Predictor Of Natural Disordered Regions (PONDR) predictor, VSL2, VL3, VL-XT and XL1-XT (Xue et al., 2010), and repeated regions of OsREPRPs were further analyzed by RADAR software to reveal super-repeat segments.

Recombinant Protein Purification

The sequences of OsRePRP1.1 from 23 to 360 aa and OsRePRP2.1 from 19 to 247 aa were amplified with the primer pairs RE-OCP-GAP-F/RE-OCP-GAP-F and OSR2.1-GAP-F/OSR2.1-GAP-R, respectively, and cloned into the expression vector pGAPZaC by using 5'-EcoR1/NotI-3’ sites. Recombinant OsRePRP1.1 and OsRePRP2.1 proteins were expressed in Pichia pastoris SMD1168 according to the manufacturer’s manual (Invitrogen). The serial segments OsRePRP1.151-159, OsRePRP1.151-131, OsRePRP1.151-105, OsRePRP1.1106-131, OsRePRP2.160-179, OsRePRP2.160-132, OsRePRP2.188-132, and OsRePRP2.160-87 were amplified with the primer pairs YSC5/YSC6, YSC21/YSC22, YSC5/YSC9, YSC10/YSC11, YSC12/YSC13, YSC12/YSC15, YSC17/YSC18, and YSC12/YSC16, respectively, and cloned into the expression vector pET28a by using 5’-XhoI/EcoR1-3’ sites. The segments OsRePRP1.151-131 and OsRePRP2.188-132 were cloned into the pET20b and pColdII vectors by using 5’-NdeI/XhoI-3’ and 5’-XhoI/EcoR1-3’ sites, respectively. All rOsRePRP1.1 and rOsRePRP2.1 segments were expressed with His-Tag in Escherichia coli, purified by using Ni Sepharose (GE) and His-Tag was not removed. The primer sequences for constructs and induction conditions for the recombinant protein expression in E. coli are listed in Supplemental Tables 4 and 5, respectively. Full-length rOsRePRP1.1 and rOsRePRP2.1 used for AG binding assays were constructed and purified as described (Tseng et al., 2013). Purified recombinant
proteins were dialyzed in 50 mM HEPES (pH 7.5) and quantified by the standard Bradford assay (Bio-Rad).

**Microscale Thermophoresis Binding Assays**

The binding affinity of purified rOsRePRPs to rabbit muscle actin (AKL-99, Cytoskeleton), porcine brain tubulin (T240, Cytoskeleton) and arabinogalactan (AG; 10830, Sigma) was measured by use of microscale thermophoresis with the Monolith NT.115 instrument (Nanotemper Technologies). For actin and tubulin binding, a serial dilution of rOsRePRPs was incubated with 1-2 nM NT-647-labeled actin or tubulin for 10 min in the assay buffer (50 mM HEPES, 0.05% (v/v) Tween 20, pH 7.5). For AG binding, a serial dilution of AG was incubated with 3 nM NT-647-labeled rOsRePRPs for 10 min in the assay buffer (50 mM HEPES, 150 mM NaCl, 5 mM CaCl$_2$, 1 mM MgCl$_2$, 0.05% Tween 20, pH 7.5). The sample was loaded into the NanoTemper glass capillaries, and microthermophoresis involved 20% LED and 20% MST power. $K_d$ was calculated by using the mass action equation with the NanoTemper software in triplicate experiments.

**Plant Materials and Growth Conditions**

The rice cultivar *Oryza sativa* cv. Tainung 67 was used throughout this study. Seeds of wild-type and transgenic rice were sterilized with 2% sodium hypochlorite for 20 min, washed thoroughly with distilled water, soaked in distilled water at 28°C for 2 days in darkness, then germinated in Petri dishes containing water with or without hygromycin B (25 µg/mL) at 28°C for 3 days. Three-day-old seedlings were transferred and cultivated in a beaker containing half-strength Kimura B solution (Baba and Takahashi, 1956). The hydroponically cultivated seedlings were grown at...
28°C and 90% relative humidity in a 14-hr-light/10-hr-dark condition. Transgenic rice plants of OsRePRP2-OX, OsRePRP-Ri, 35Spro::GFP, Ubipro::OsRePRP1-GFP, Ubipro::OsRePRP2-GFP and OsRePRP2.1pro::GUS were generated as previously described (Tseng et al., 2013).

Rice Root Protoplast Transfection

The expression vectors of pSAT6-EGFP-C1/N1 and pSAT6-DsRed-C1/N1 were used in the rice root protoplast transient-expression system (Tzfira et al., 2005). Plasmids containing LifeAct-GFP with OsRePRP2-DsRed or DsRed and GFP-MBD with OsRePRP2-DsRed or DsRed were generated. The 51-bp LifeAct with the GGSG linker were produced by mixing the primer pair HAS106/HAS107 at room temperature for 30 min. LifeAct was further PCR-amplified with the primer pair HAS108/HAS109 and cloned into pSAT6-EGFP-N1 with restriction enzymes NcoI and BamHI. The 1254-bp MBD (2048-3661 bp in mouse MAP4 cDNA) was amplified with the primer pair HAS140/HAS141 from the cDNA of C57BL/6 mice, which was a gift from Dr. Ya-Lin Lin (Institute of Biomedical Sciences, Academia Sinica), and cloned into pSAT6-EGFP-C1 with restriction enzymes HindIII and EcoRI. The 741-bp OsRePRP2 amplified by the primer pair HAS126/HAS127 was cloned into pSAT6-DsRed-N1. The 35Spro::OsRePRP2-DsRed and 35Spro::DsRed fragments amplified with the primer pair HAS142/HAS137 were further cloned into plasmids containing LifeAct-GFP and GFP-MBD, by using Ndel. Primer sequences are given in Supplemental Table S4. The isolation of rice root protoplasts followed the published protocol with slight modifications (Evrard et al., 2012). Whole roots harvested from 5-day-old rice seedlings were chopped in digestion buffer (400 mM mannitol, 20 mM MES hydrate, 20 mM KCl, 1.1% Cellulase R10, 0.9% Cellulase RS, 0.3% Macerozyme R10, 0.12% Pectolyase Y-23, 10 mM CaCl2, 0.1% BSA, 2.5 mM
β-mercapoethanol, pH 5.7), vacuumed for 7 min, then incubated for 4 hr with 80-rpm shaking at the room temperature. The solution was filtered through a 40-μm cell strainer and protoplasts were harvested by centrifugation at 500 x g for 5 min. Protoplasts were washed and resuspended in the WI buffer (400 mM mannitol, 20 mM MES hydrate, 20 mM KCl, pH 5.7). The concentration of protoplasts was adjusted to 800,000 protoplasts per milliliter by using a hemocytometer. The transfection of rice root protoplasts involved the PEG-mediated method (Yoo et al., 2007). About 15 μg plasmid DNA in 10 μL was mixed with 100 μL protoplasts and 110 μL PEG solution (40% PEG 4000, 0.2 M mannitol, 0.1 M CaCl₂) and incubated at room temperature for 15 min. The transformed protoplasts were incubated in WI buffer for 16-24 hr in the dark before observation.

**Fluorescence Imaging in Rice Root Protoplasts and Analysis**

The 3.5-μL protoplast suspension was placed in a well created by a 6-mm hollow circle tap pasted on slides and observed under a Zeiss LSM 880 confocal laser scanning microscope with a 40× water objective (N.A. of 1.2). Fluorescence images were taken in the Airyscan superresolution mode and DIC images were taken in the standard confocal mode. Samples were excited by using an argon ion laser at wavelength 488 nm for GFP and a HeNe ion laser at 561 nm for DsRed. A 488/561/633 dichroic beam splitter was used to detect fluorescence; GFP fluorescence was detected with a 495- to 550-nm band-pass filter and DsRed fluorescence was detected with a 570- to 620-nm band-pass filter. For co-localization assays, the laser power for GFP 488 nm was 5% and for DsRed 561 nm was 40%. Image analysis was undertaken by using ImageJ and the Coloc 2 plugin (Arena et al., 2017) to calculate Pearson's R correlation values. All the Pearson's R correlation values were measured over the entire protoplast with no region of interest (ROI) selection by hand or by
thresholding involved. For the LifeAct-GFP with DsRed transfection control observation in wild-type, OsRePRP2-OX and OsRePRP-Ri protoplasts, images were taken under identical settings: 2% GFP 488-nm laser power, 10% DsRed 561-nm laser power, stack scanning mode, zoom 6, and image size for x: 34.23 µm, y: 34.23 µm, z: 5.00 µm with 20 sections of 0.25-µm Z scaling. For GFP-MBD with DsRed transfection control observation in wild-type, OsRePRP2-OX and OsRePRP-Ri protoplasts, the laser power for GFP 488 nm was 3% and for DsRed 561 nm was 40%.

Z-scaling data sets are shown in Supplemental Figures S12-S14)

**Actin Filament Phalloidin Staining and F-actin Quantification**

F-actin staining was performed as described (Yang et al., 2011). For F-actin observation, we used 8-day-old seedlings (control) and 7-day-old seedlings treated with 2 µM ABA for 1 day (ABA). Root tips of 1-cm segments were cut and incubated in PME buffer (100 mM PIPES, 10 mM EGTA and 5 mM MgSO₄, pH 6.8) containing 300 µM m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), 1.5% glycerol and 0.1% Triton X-100 with gentle shaking for 30 min. Samples were washed twice with PME buffer, then fixed in PME buffer containing 2% paraformaldehyde for 30 min. After rising thoroughly in PME buffer, samples were stained with actin-staining buffer [PME, 1.5% glycerol, 0.1% Triton X-100 and 66 nM Alexa Fluor 488-phalloidin (A12379, Thermo Fischer Scientific)] at 4°C in the dark overnight, then washed three times in PME buffer before confocal laser microscopy observation. Images were obtained with a 63× water objective (numerical aperture [N.A.] of 1.2) using a Zeiss LSM 880 confocal laser scanning microscope. Fluorescence was excited at 488 nm and collected with a 492-560 nm filter. The overall fluorescence signal of each genotype was obtained under identical staining conditions and confocal settings 3% 488-nm laser power, 54-µm pinhole, stack scanning mode, zoom 3, and image.
size for x: 45 µm and y: 45 µm with 10-40 sections of 0.42-µm Z scaling. Thirty images of at least three independent transgenic lines for each genotype were processed to determine average fluorescence intensity with ImageJ.

**Immunofluorescence Staining of Microtubules**

We used 6-day-old seedlings (control) and 4-day-old seedlings treated with 2 µM ABA for 2 days (ABA) for microtubule assays as described (Deng et al., 2015). Root tips of 1-cm segments were cut and fixed in 4% paraformaldehyde in PME buffer 1 (50 mM PIPES, 2 mM MgSO$_4$, 2 mM EGTA, pH 6.9) containing 0.05% Triton X-100 for 30 min. After washing thoroughly with PME buffer 1, samples were digested with 2% cellulase R-10 and 1% pectolyase Y-23 (both Yakult Pharmaceutical Industry) in PME buffer 1 at 37°C for 30 min to 1 hr. The softened root tips were washed gently with PME buffer 1 and frozen and thawed twice in liquid nitrogen. Samples were treated with the blocking buffer [3% BSA in PBST (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.76 mM KH$_2$PO$_4$, 0.05% Triton X-100)] for 1 hr at room temperature, then incubated with 1/50 diluted primary antibodies anti-α-tubulin (T9026, Sigma) in the blocking buffer at 4°C overnight. After washing with PBST for 5 times, samples were incubated with 1/800-diluted secondary antibodies Alexa Fluor 488 goat anti-mouse IgG (A-11001, Thermo Fischer Scientific) in PBST at 37°C for 3 hr. After washing 4 times with PBST and once with PBS, samples were mounted with PBS containing 50% glycerol and 0.1% o-phenylenediamine. Images were obtained with a 63× water objective (N.A. of 1.2) under a Zeiss LSM 880 confocal laser scanning microscope. Fluorescence was excited at 488 nm and collected with a 492-560 nm filter. The overall fluorescence signal of each genotype was obtained under identical staining conditions and confocal settings 8% 488-nm laser power, 58-µm pinhole, and stack scanning mode.
**Immunogold Electron Microscopy**

Segments of 3-day-old root tips were cut and frozen in a high-pressure freezer (Leica EM PACT2) at 20,000 to 20,050 MPa. Freeze substitution was conducted in anhydrous acetone solution containing 0.1% uranyl acetate and 0.2% glutaraldehyde with an automatized Leica EM AFS2 system. Samples were successively kept at -85°C for 3 days, -60°C for 1 day, -20°C for 1 day, 0°C for 1 day, and at room temperature. After two acetone changes, root segments were infiltrated and embedded in LR Gold Resin. Ultrathin sections of 90 to 120 nm were cut by using a Reichert Ultracut S or Leica EM UC6 microtome (Leica) and collected on 100-mesh nickel grids, which were floated on PBS for 15 min, then on PBS and 1% BSA for 15 min. The grids were incubated with primary antibodies 1/50-diluted rabbit anti-GFP (ab6556, Abcam) and 1/20-diluted mouse anti-α-tubulin (T9026, Sigma) or mouse anti-actin (A0480, Sigma) for 1 hr at room temperature. After 4 washes with PBS, grids were incubated with 12-nm/18-nm Colloidal Gold Donkey anti-rabbit/anti-mouse IgG (Jackson ImmunoResearch) at room temperature for 1 hr, then washed four times with PBS and three times with distilled water. After immunogold labeling, sections were stained with 5% uranyl acetate for 10 min and 0.5% lead citrate for 4 min. Sections were observed under a Tecnai G² Spirit transmission electron microscope (FEI, Hillsboro) at 80 KV, and images were obtained with a Gatan Orius CCD camera. The distance from the 18-nm gold particle to the closest 12-nm gold particle was calculated by using ImageJ, and quantification of the immunogold TEM data was performed as described (Bergersen et al., 2008).

**Metabolic Click-labeling**

Fucose alkyne incorporation and fluorescent labeling were performed as described...
with slight modifications (Anderson et al., 2012). Seven-day-old seedlings were transferred to half-strength Kimura B solution containing 10 μM fucose alkyne (C10264, Thermo Fischer Scientific) and incubated for 24 hr under the growth conditions described previously, then washed three times and transferred to labeling solution (half-strength Kimura B solution containing 1 mM CuSO₄, 1 mM ascorbic acid and 0.4 μM Alexa Fluor 488-azide [A10266, Thermo Fischer Scientific]) for labeling at 28°C in the dark for 2 hr. Confocal laser microscopy observation was obtained with a 40× water objective (N.A. of 1.2) using a Zeiss LSM 880 confocal laser scanning microscope. Fluorescence was excited at 488 nm and collected with a 492-560 nm filter. The overall fluorescence signal of each genotype was obtained under identical staining conditions and confocal settings 3% 488-nm laser power, 58-μm pinhole, stack scanning mode, zoom 3, and image size for x: 45 μm and y: 45 μm with 30 sections of 0.45-μm Z scaling. Thirty images of at least three independent transgenic lines for each genotype were processed to determine average fluorescence intensity with ImageJ.

Cell Wall Texture Observation

For cell-wall cellulose microfibril observation, 8-day-old seedlings (control) and 4-day-old seedlings treated with 2 μM ABA for 4 days (ABA) were used. Cell-wall preparation was as described (Sugimoto et al., 2000) with slight modifications. The whole roots were cryoprotected in PME buffer 2 (25 mM PIPES, 0.5 mM MgSO₄, 2.5 mM EGTA, pH 7.2) containing 25% and 50% DMSO for 10 min for each step. Root tips were excised, placed on sample carriers and cut by using a glass knife on a Leica Ultracut UCT ultramicrotome equipped with the Leica EM FCS cryo-chamber attachment at -120°C. The remaining root strips were thawed in PME buffer 2 containing 50% DMSO, then transferred to PME buffer 2. Samples were treated with
acetic acid and nitric acid and distilled water (8:1:2) for 1 hr at 95°C. After a thorough washing in distilled water, samples were dehydrated with an ethanol series (30%, 50%, 70%, 95% and 100% three times, 30 min for each step), critical point dried with CO₂ and further mounted on carbon tape-covered stubs with the cut surface facing upward, and coated with carbon after platinum at 20 mA for 80 sec. The cell-wall fine structure was examined with a FE-SEM JSM-7100F microscope (JEOL) fitted with an accelerated voltage 5 kV, probe current 0.1 nA and working distance 5 to 6 mm. The quantitative directionality analysis involved using the Fiji package (Schindelin et al., 2012). The Fourier component method was used and orientation from -90° to 90° was analyzed by using the Fiji plug-in “Directionality” following the instructions.

**Starch Staining and Quantification**

For starch staining, 14-day-old seedlings (control) and 14-day-old seedlings treated with 20% PEG6000 for 1 hr (PEG) were used. Rice roots were stained in 1/10 diluted 5% Lugol's iodine solution for 10 min, destained with distilled water for 30 min and the root architecture images were captured by using an Epson scanner. For sectioning, 1-cm root segments were embedded in 5% agar and cut into 100- to 120-μm sections using a DTK-100 microslicer (Dosaka EM). The sections were stained with iodine and observed under a Zeiss Axio Imager Z1 microscope. For starch quantification, 14-day-old seedlings (control) and 14-day-old seedlings treated with 20% PEG6000 for 5 hr (PEG) were used. Whole roots of 10 to 20 seedlings were ground in liquid nitrogen with a pestle and mortar and dried at 65°C, then dry weight was measured. A starch colorimetric/fluorometric assay kit (K647, Biovision) was used to determine the level of starch.
Enzyme Activity Assays and PhosTag Western Blot Analysis

Whole roots of 10 to 20 14-day-old seedlings were ground into a fine powder in liquid nitrogen by using a pestle and mortar, mixed with 1-mL ice-cold extraction buffer (100 mM HEPES-KOH, pH 7.5, 5 mM MgCl₂, 5 mM DTT), and centrifuged at 11,000 x g for 15 min at 4°C. The supernatant was then collected and used for the measuring sucrose synthase enzyme activity in the cleavage direction according to Sun et al. (1992). The protein extract was quantified by using the Bio-Rad Protein Assay Reagent, adjusted to 1 mg/mL, mixed with the reaction mixture (50 mM HEPES-KOH, pH 7, 2 mM MgCl₂, 15 mM KCl, 50 mM sucrose, 5 mM UDP, 0.5 mM NADP, 1 mM PPI, 1 U/mL phosphoglucomutase [P3397, Sigma], 2 U/mL glucose-6-P dehydrogenase [G8404, Sigma], and 0.5 U/mL UDP-glucose pyrophosphorylase [U8501, Sigma]), and incubated at 30°C for 30 min. All control reactions lacked sucrose and UDP. The reaction was terminated by heating for 5 min in a boiling water bath. NADPH production was monitored at 340 nm with a SYNERGY H1 microplate reader (BioTek). Sucrose synthase enzyme activity in the synthetic direction was assayed by using the Sucrose Synthase Microplate Assay Kit (MBS8243224, MyBioSource). The formation of UDP-glucose-dependent sucrose was monitored at 480 nm with a SYNERGY H1 microplate reader (BioTek).

For anti-SUS western blot analysis with/without PhosTag, whole roots of 14-day-old seedlings were ground, mixed with the extraction buffer (20 mM sodium phosphate pH 7.4, 50 mM β-glycerophosphate, 100 μM Na₃VO₄, 1 mM PMSF, 1× protease inhibitor cocktail) and centrifuged at 12,000 x g for 1 hr at 4°C. The protein extract supernatant was quantified by using Bio-Rad Protein Assay Reagent, and 2.5 µg of protein was separated in 6% polyacrylamide gels. For PhosTag western blot analysis, separating gel contained 50 µM Zn(NO₃)₂ and 50 µM PhosTag and the running buffer
consisted of 100 mM Tris, 100 mM MOPS, 0.1% SDS and 5 mM NaHSO₃ (pH 7.8). The electrophoresis was performed at constant 10 mA at 4°C for 10 hr. After a washing in the transfer buffer (25 mM Tris-HCl pH 7.4, 192 mM glycine) containing 10 mM EDTA, the gel was transferred to the PVDF membrane with the transfer buffer containing 5 % methanol and 1 mM EDTA at 24V overnight. Immunoblot analysis was performed with 1/25000 diluted anti-SUS antibodies (AS152830, Agrisera). Signals were detected with ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences). The band intensity of PhosTag gel was not estimated by scanning.

**Accession numbers**

Sequence data from this article can be found in the rice RGAP database (http://rice.plantbiology.msu.edu/) with the following accession numbers:

- OsRePRP1.1 (LOC_Os05g13900), OsRePRP1.2 (LOC_Os05g13940), OsRePRP2.1 (LOC_Os07g23660), OsRePRP2.2 (LOC_Os07g23640), OsSUS1 (LOC_Os03g28330), OsSUS2 (LOC_Os06g09450), OsSUS3 (LOC_Os07g42490), OsSUS4 (LOC_Os03g22120), OsSUS5 (LOC_Os04g24430), OsSUS6 (LOC_Os02g58480), OsRBG1 (LOC_Os11g30430), OsEF1α (LOC_Os03g08020), OsGADPH (LOC_Os04g40950), and OsUBQ5 (LOC_Os01g22490). The accession number of HvHVA1 in GenBank is X78205.

**Supplemental Data**

Supplemental Figure S1. Disorder prediction and internal duplication of OsRePRPs.

Supplemental Figure S2. Segmental repeat organization of OsRePRPs.

Supplemental Figure S3. *In Vitro* binding assays of OsRePRPs.
Supplemental Figure S4. Co-immunoprecipitation assays and immunogold transmission electron microscopy (TEM) of GFP, actin and tubulin.

Supplemental Figure S5. The expression of OsRePRP2 in rice roots under control and ABA treatments.

Supplemental Figure S6. OsRePRP is necessary and sufficient in ABA repression of root elongation, affecting F-actin organization.

Supplemental Figure S7. OsRePRP affects F-actin organization in division and differentiation zones.

Supplemental Figure S8. OsRePRP affects F-actin organization under PEG treatment.

Supplemental Figure S9. Starch accumulation in rice roots after PEG treatment.

Supplemental Figure S10. qPCR analysis of OsRePRP expression.

Supplemental Figure S11. Subcellular localization of OsRePRP2 in rice root protoplasts.

Supplemental Figure S12. Rice root protoplasts co-expressing LifeAct-GFP and OsRePRP2-DsRed.

Supplemental Figure S13. Rice root protoplasts co-expressing GFP-MBD and OsRePRP2-DsRed.

Supplemental Figure S14. Rice root protoplasts expressing LifeAct-GFP.

Supplemental Table S1. Amino acid sequences of actin peptide hits by LC-MS/MS.

Supplemental Table S2. Amino Acid Sequences of tubulin peptide hits by LC-MS/MS.

Supplemental Table S3. Amino acid sequences of sucrose synthase peptide hits by LC-MS/MS.

Supplemental Table S4. Primer sequences used in plasmid construction.
Supplemental Table S5. Induction Conditions of Recombinant Protein Expression in *Escherichia coli*.

Supplemental Table S6. Primer Sequences Used in qRT-PCR analysis.

Supplemental Video

**Acknowledgments**

The authors thank Dr. Shu-Chuan Chris Jao and Szuhuan Wang of the Biophysics Core Facility, Academia Sinica (AS), for assistance in operating the Monolith NT.115 pico instrument. Peptides were synthesized in the Institute of Biological Chemistry, AS. Confocal images, immunogold TEM, and LC-MS/MS analysis were conducted in the Live-Cell-Imaging Core Lab, Plant Cell Biology Core Lab, and Proteomics Core Lab, respectively. Cryosection and FE-SEM were done at the Imaging Core in the Institute of Cellular and Organismic Biology and the Electron Probe Micro-Analysis Lab in the Institute of Earth Sciences, AS, respectively. We acknowledge the *In situ* Hybridization Core Facility of the Agricultural Biotechnology Research Center, AS, for performing the *in situ* experiments. We thank the technical assistance of Dr. Yoshiyuki Iizuka, Yu-Shiang Wang, Dr. Bo-Yan Lin, Yao-Kuan Huang, Simon Wei and critical comment of the manuscript by Prof. Edward Chee-Tak Yeung and Dr. Shiu-Cheung Lung.
Figure legends

Figure 1. In Vitro Interactions of Recombinant OsRePRP2.1 (rOsRePRP2.1) and Actin/Tubulin.

(A) Segmental repeat organization of OsRePRP2.1. The diagram shows the full-length amino acid (aa) sequences analyzed by RADAR (http://www.ebi.ac.uk/Tools/pfa/radar/). The repeat modules of OsRePRP2.1 are represented as blue and pink blocks and the non-repeat regions are white blocks. The percentage of the amino acid sequences are distinguished by colors of one-letter codes, red for 100%, green for >75%, blue for >67%, black for >50% and dash for non-consensus amino acids. (B) The binding affinity of rOsRePRP2.1 with actin/tubulin. In vitro binding assays were conducted with microscale thermophoresis (MST) and the binding affinity is represented as dissociation constant \( K_d \) values. Asterisks indicate the binding affinity with synthetic peptides. (C-F) Binding isotherms for rOsRePRP2.1\(^{60-179}\)(C-D) and synthetic peptides OsRePRP2.1\(^{60-87}\) (E-F) to rabbit muscle actin (C, E) and porcine brain tubulin (D, F). Rabbit muscle actin and porcine brain tubulin were fluorescently labeled with AlexaFluor 647 and kept at a constant concentration of 1.2-1.5 nM. The binding of OsRePRP2.1 alone to actin and tubulin is represented as blue and green curves, respectively, and the binding of OsRePRP2.1 in the presence of AG is represented as pink-dashed curves. Closed circles indicate rOsRePRP2.1\(^{60-179}\) alone (C-D) and synthetic peptides OsRePRP2.1\(^{60-87}\) alone (E-F). Open triangles indicate rOsRePRP2.1\(^{60-179}\) (C-D) and OsRePRP2.1\(^{60-87}\) (E-F) in the presence of 250 \( \mu \)M arabinogalactan (AG). Closed squares indicate rOsRePRP2.1\(^{60-179}\) and synthetic peptides OsRePRP2.1\(^{60-87}\) in the presence of 25 \( \mu \)M porcine brain tubulin (C, E) or 57.5 \( \mu \)M rabbit muscle actin (D, F). Data are mean±SD of three technical repeats. G-actin and \( \alpha \beta \)-tubulin dimers were used, and at least two independent batches of recombinant proteins or synthetic peptides were subjected to the binding assays.

Figure 2. Interactions between OsRePRP2 and Actin and Tubulin in Rice Root Protoplasts.

Five-day-old wild-type rice root protoplasts co-expressing LifeAct-GFP and OsRePRP2-DsRed (A-D), GFP-MBD and OsRePRP2-DsRed (E-H), LifeAct-GFP and DsRed (I-L), and GFP-MBD and DsRed (M-P). GFP and DsRed signals are shown in green (A, E, I, M) and magenta (B, F, J, N); overlay of green and red channels are in (C, G, K, O), and the DIC channel indicating bright field images in (D, H, L, P). Five-day-old wild-type rice root protoplasts expressing LifeAct-GFP (Q-R) and GFP-MBD (S-T), GFP signals are shown in green (Q, S) while the respective control images in the DsRed channel are shown in (R, T). The Pearson's R correlation
coefficients calculated by Pearson coefficient analysis are shown in a box plot (U). The Pearson's R coefficients for LifeAct-GFP and OsRePRP2-DsRed, GFP-MBD and OsRePRP2-DsRed, LifeAct-GFP and DsRed, and GFP-MBD and DsRed are shown in green, blue, magenta and black boxes, respectively (U). The line inside the box indicates the median, and the cross indicates the mean. Box edges are the 25th to 75th percentiles; whiskers indicate the range and an outlying data point is shown as a circle. Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Data are measured from 15 protoplasts of three biological experiments. The Pearson's R correlation coefficients for each comparison are shown in (C, G, K, O). Bar = 2 μm.

Figure 3. Interactions between OsRePRP and Actin and Tubulin in Planta.

(A-B, E-G) Double immunogold-labeling transmission electron microscopy of root elongation zone cells of 3-day-old OsRePRP1-GFP (A-B) and OsRePRP2-GFP (E-G) rice. The smaller 12-nm gold particles represent the distribution of actin (A, E, G) and tubulin (B, F), and the larger 18-nm gold particles represent the distribution of OsRePRP1-GFP (A-B) and OsRePRP2-GFP (E-G). Arrows indicate co-localization of OsRePRP-GFP and actin (A, E, G) or tubulin (B, F). (C-D, H-I) The distances from each OsRePRP-GFP gold particle to the closest actin or tubulin gold particle were measured as described in Methods. The bars represent the distribution of inter-gold-particle-center distances between OsRePRP-GFP and actin or tubulin ranging from 0 nm to > 100 nm. Ten images were measured, and two biological replicates were performed. Bar = 100 nm in A-B, E-G.

Figure 4. OsRePRP Affects F-actin Organization.

(A-F) F-actin organization in the wild-type (WT), OsRePRP2-OX and OsRePRP-Ri root cells visualized by Alexa Fluor 488-phalloidin staining. The epidermal cells of the root elongation zones of 8-day-old seedlings (control; A-C) and 7-day-old seedlings treated with 2 μM ABA for 1 day (ABA; D-F) were observed. (G) Quantitative analysis of F-actin level in WT, OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) 8-day-old seedlings (control) and 7-day-old seedlings treated with 2 μM ABA for 1 day (ABA). Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Data are mean±SD fluorescence intensity of 30 images. More than six biological replicates were performed and at least three independent transgenic lines for each genotype were observed (OsRePRP2-OX #2, #10, #19, #24 and OsRePRP-Ri #5, #6, #7). Bar = 10 μm.

Figure 5. OsRePRP Affects F-actin Organization in Root Protoplasts.
The transient expression of LifeAct-GFP in 5-day-old rice root protoplasts from the wild type (A, D), OsRePRP2-OX (B, E) and OsRePRP-Ri (C, F). GFP signals are shown in green (A-C) and the DIC channel indicates bright field images in (D-F). Images in (A-C) represent the maximum intensity projection of 20 optical confocal images with identical settings. Three independent transgenic lines for each genotype were observed (OsRePRP2-OX #2, #10, #24 and OsRePRP-Ri #5, #6, #7). Bar = 2 μm.

Figure 6. OsRePRP Affects Microtubule Organization.

(A-C) Micrographs of microtubule staining in WT (A), OsRePRP2-OX (B) and OsRePRP-Ri (C) root cells of elongation zones. (D-F) Micrographs of microtubule staining in WT (D), OsRePRP2-OX (E) and OsRePRP-Ri (F) root cells after 2 μM ABA treatment for 2 days. More than three biological replicates were performed and three independent transgenic lines for each genotype were observed (OsRePRP2-OX #10, #19, #24 and OsRePRP-Ri #5, #6, #7). (G-L) The transient expression of GFP-MBD in 5-day-old rice root protoplasts from the WT (G, J), OsRePRP2-OX (H, K) and OsRePRP-Ri (I, L). GFP signals are shown in green (G-I) and the DIC channel indicates bright field images in (J-L). Four biological replicates were performed and three independent transgenic lines for each genotype were observed (OsRePRP2-OX #2, #10, #24 and OsRePRP-Ri #5, #6, #7). Bar = 2 μm.

Figure 7. OsRePRP Affects Non-cellulosic Polysaccharide Secretion and Cell-Wall Cellulose Microfibril Network.

(A-C) Localization of fucose alkyne (FucAl) incorporation in WT (A), OsRePRP2-OX (B) and OsRePRP-Ri (C) root elongation zone cells. Images were obtained with identical settings including laser power, pinhole, objective, zoom, and channel/filter wavelengths as described in Methods with no image contrast or brightness corrections. (D) Quantitative analysis of FucAl-associated fluorescence signal in the WT, OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) are shown in a box plot. The line inside the box indicates the median, and the cross indicates the mean. Box edges are the 25th to 75th percentiles; whiskers indicate the range. Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Data are mean/median (Q1-Q3) fluorescence intensity of 30 images. More than four biological replicates were performed and at least three independent transgenic lines for each genotype were observed (OsRePRP2-OX #2, #10, #19, #24 and OsRePRP-Ri #5, #6, #7). (E-J) Field-emission scanning electron microscopy images are oriented to show cell-wall cellulose microfibril alignment relative to the cell and the root long axis. (E-G) Micrographs of cell-wall cellulose microfibril network in WT (E), OsRePRP2-OX
(F) and OsRePRP-Ri (G) root elongation zone cells under the control condition. (H-J) Micrographs of cell-wall microfibril network in WT (H), OsRePRP2-OX (I) and OsRePRP-Ri (J) root elongation zone cells after 2 μM ABA treatment for 4 days. Three biological replicates were performed and at least two independent transgenic lines for each genotype were observed (OsRePRP2-OX #10, #19, #24 and OsRePRP-Ri #5, #6). (K-L) Directionality histograms of cell wall cellulose microfibril network under the control condition (K) or after ABA treatment (L). Directionality analysis of WT (black line), OsRePRP2-OX (magenta line) and OsRePRP-Ri (blue line) using the Fiji package (Schindelin et al., 2012) as described in Methods. Data are mean frequency of fibers in specific orientation analyzed from 15 images. (M) Relative density of root segments in WT, OsRePRP2-OX and OsRePRP-Ri. Two-cm root-tip segments from 12-day-old seedlings (control) and 8-day-old seedlings treated with 2 μM ABA for 4 days (ABA) were cut and immersed in the distilled water. Six biological replicates were performed and three independent transgenic lines for each genotype were observed (OsRePRP2-OX #10, #19, #24 and OsRePRP-Ri #5, #6, #7). (N) Quantitative analysis of total root dry weight (mg) divided by mean root length (cm) per seedling. Whole roots were harvested from WT, OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) 14-day-old seedlings (control) and 8-day-old seedlings treated with 2 μM ABA for 6 days (ABA). Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Fluorescne intensity of 30 images was measured. Three independent transgenic lines for each genotype were measured (OsRePRP2-OX #2, #10, #24 and OsRePRP-Ri #5, #6, #7). Bar = 2 μm in (A-C), 100 nm in (E-J), 1 cm in (M).

Figure 8. Starch Accumulation in Rice Roots after PEG Treatment.

(A-B) Rice roots from 14-day-old seedlings of WT, OsRePRP2-OX and OsRePRP-Ri under control (A) or PEG (B) treatments stained with iodine. Squares in (A-B) represented the sectioning parts in (C-Q). Four biological replicates were performed and at least two independent transgenic plants for each genotype were observed (OsRePRP2-OX #2, #24 and OsRePRP-Ri #5, #6, #7). (C-Q) Cross and longitudinal root sections of WT (C, F, I, L, O), OsRePRP2-OX (D, G, J, M, P) and OsRePRP-Ri (E, H, K, N, Q) under control (C-K) or PEG (L-Q) treatments stained with iodine. Three biological replicates were performed and at least two independent transgenic lines for each genotype were observed (OsRePRP2-OX #10, #19, #24 and OsRePRP-Ri #5, #7). (R) Starch content measurements in the whole roots of WT, OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) under control or PEG treatments. Significant differences are indicated with asterisks (P < 0.05, two-tailed Mann-Whitney U test). Data are mean±SD of six technical repeats. Three biological replicates were
performed and two independent transgenic lines for each genotype were measured (OsRePRP2-OX #10, #24 and OsRePRP-Ri #5, #6). Bar = 1 cm in (A-B) and 50 \mu m in (C-Q).

Figure 9. Enzyme Activity of Sucrose Synthase (SUS) Was Changed in OsRePRP2-OX.

(A) Co-immunoprecipitation assays of GFP, OsRePRP1-GFP and OsRePRP2-GFP transgenic rice. Total protein was extracted from whole roots of 4-day-old seedlings treated with 2 \mu M ABA for 2 days. Western blot analysis involved anti-SUS antibodies. The arrow indicates 93-kDa SUS. Two biological replicates were performed. (B-C) Enzyme activity of sucrose synthase in the cleavage (B) and synthetic (C) directions. Total protein extract from 14-day-old whole roots of the wild type (WT), OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) was shown in box plots. The line inside the box indicates the median, and the cross indicates the mean. Box edges are the 25\textsuperscript{th} to 75\textsuperscript{th} percentiles; whiskers indicate the range. Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Nine repeats from three independent biological replicates were performed. (D) PhosTag western blot (WB) and WB analysis with anti-SUS antibodies. Total protein extract from 14-day-old whole roots of the wild type (WT), OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) was assayed. Four biological replicates were performed.
Literature Cited

al-Habori M (1995) Microcompartmentation, metabolic channelling and carbohydrate metabolism. Int J Biochem Cell Biol 27: 123–132

Anderson CT, Wallace IS, Somerville CR (2012) Metabolic click-labeling with a fucose analog reveals pectin delivery, architecture, and dynamics in Arabidopsis cell walls. Proc Natl Acad Sci USA 109: 1329–1334

Arena ET, Rueden CT, Hiner MC, Wang S, Yuan M, Eliceiri KW (2017) Quantitating the cell: turning images into numbers with ImageJ. Wiley Interdiscip Rev Dev Biol 6: e260

Baba I, Takahashi Y (1956) Water and sand culture methods. In: Togari Y, Matsuo T, Hatamari M, Yamada N, Harada T, Suzuki N, editors. Experimental methods in crop science. Tokyo: Association of Agricultural Techniques p157–185

Balasubramanian R, Karve A, Kandasamy M, Meagher RB, Moore Bd (2007) A role for F-actin in hexokinase-mediated glucose signaling. Plant Physiol 145: 1423–1434

Baluska F, Hlavacka A, Samaj J, Palme K, Robinson DG, Matoh T, McCurdy DW, Menzel D, Volkmann D (2002) F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. Plant Physiol 130: 422–431

Baluska F, Samaj J, Wojtaszek P, Volkmann D, Menzel D. (2003) Cytoskeleton-plasma membrane-cell wall continuum in plants. Emerging links revisited. Plant Physiol 133: 482–491

Bashline L, Lei L, Li S, Gu Y (2014) Cell wall, cytoskeleton, and cell expansion in higher plants. Mol Plant 7: 586–600

Baskin TI (2005) Anisotropic expansion of the plant cell wall. Annu Rev Cell Dev Biol 21: 203–222
Baskin TI, Meekes HT, Liang BM, Sharp RE (1999) Regulation of growth anisotropy in well-watered and water-stressed maize roots. II. Role of cortical microtubules and cellulose microfibrils. Plant Physiol 119: 681–692

Bergersen LH, Storm-Mathisen J, Gundersen V (2008) Immunogold quantification of amino acids and proteins in complex subcellular compartments. Nat Protoc 3: 144–152

Boothby TC, Tapia H, Brozena AH, Piszkiewicz S, Smith AE, Giovannini I, Rebecchi L, Pielak GJ, Koshland D, Goldstein B (2017) Tardigrades Use Intrinsically Disordered Proteins to Survive Desiccation. Mol Cell 65: 975–984

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2: 48–54

Buckley TN (2019) How do stomata respond to water status? New Phytol 224: 21–36

Candat A, Paszkiewicz G, Neveu M, Gautier R, Logan DC, Avelange-Macherel MH, Macherel D (2014) The ubiquitous distribution of late embryogenesis abundant proteins across cell compartments in Arabidopsis offers tailored protection against abiotic stress. Plant Cell 26: 3148–3166

Chuong SD, Good AG, Taylor GJ, Freeman MC, Moorhead GB, Muench DG (2004) Large-scale identification of tubulin-binding proteins provides insight on subcellular trafficking, metabolic channeling, and signaling in plant cells. Mol Cell Proteomics 3: 970–983

Covarrubias AA, Cuevas-Velazquez CL, Romero-Pérez P.S, Rendón-Luna DF, Chater CCC (2017) Structural disorder in plant proteins: where plasticity meets sessility. Cell Mol Life Sci 74: 3119–3147

Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof YD, Schumacher K, Gonneau M, Höfte H, Vernhettes S (2009) Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. Plant Cell 21: 1141–1154
Deng ZY, Liu LT, Li T, Yan S, Kuang BJ, Huang SJ, Yan CJ, Wang T (2015) OsKinesin-13A is an active microtubule depolymerase involved in glume length regulation via affecting cell elongation. Sci Rep 25: 9457

Driouich A, Faye L, Staehelin LA (1993) The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. Trends Biochem Sci 18: 210–214

Duncan KA, Huber SC (2007) Sucrose synthase oligomerization and F-actin association are regulated by sucrose concentration and phosphorylation. Plant Cell Physiol 48: 1612–1623

Era A, Tominaga M, Ebine K, Awai C, Saito C, Ishizaki K, Yamato KT, Kohchi T, Nakano A, Ueda T (2009) Application of Lifeact reveals F-actin dynamics in Arabidopsis thaliana and the liverwort, Marchantia polymorpha. Plant Cell Physiol 50: 1041–1048

Evrard A, Bargmann BO, Birnbaum KD, Tester M, Baumann U, Johnson AA (2012) Fluorescence-activated cell sorting for analysis of cell type-specific responses to salinity stress in Arabidopsis and rice. Methods Mol Biol 913: 265–276

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell 14 Suppl: S15–45

Fujita S, Pytela J, Hotta T, Kato T, Hamada T, Akamatsu R, Ishida Y, Kutsuna N, Hasezawa S, Nomura Y, et al (2013) An atypical tubulin kinase mediates stress-induced microtubule depolymerization in Arabidopsis. Curr Biol 23: 1969–1978

Fukao T, Xu K, Ronald PC, Bailey-Serres J (2006) A variable cluster of ethylene response factor-like genes regulates metabolic and developmental acclimation responses to submergence in rice. Plant Cell 18: 2021–2034

Garagounis C, Kostaki KI, Hawkins TJ, Cummins I, Fricker MD, Hussey PJ, Hetherington AM, Sweetlove LJ (2017) Microcompartmentation of cytosolic aldolase by interaction with the actin cytoskeleton in Arabidopsis. J Exp Bot 68: 885–
Giarola V, Hou Q, Bartels D (2017) Angiosperm plant desiccation tolerance: hints from transcriptomics and genome sequencing. Trends Plant Sci 22: 705–717

Grossman M, Ben-Chetrit N, Zhuravlev A, Afik R, Bassat E, Solomonov I, Yarden Y, Sagi I (2016) Tumor cell invasion can be blocked by modulators of collagen fibril alignment that control assembly of the extracellular matrix. Cancer Res 76: 4249–4258

Gutierrez-Cruz G, Van Heerden AH, Wang K (2001) Modular motif, structural folds and affinity profiles of the PEVK segment of human fetal skeletal muscle titin. J Biol Chem 276: 7442–7449

Hu H, Juvekar A, Lyssiotis CA, Lien EC, Albeck JG, Oh D, Varma G, Hung YP, Ullas S, Lauring J, et al (2016) Phosphoinositide 3-Kinase Regulates Glycolysis through Mobilization of Aldolase from the Actin Cytoskeleton. Cell 164: 433–446

Hu H, Xiong L (2014) Genetic engineering and breeding of drought-resistant crops. Annu Rev Plant Biol 65: 715–741

Huber SC, Huber JL, Liao PC, Gage DA, McMichael RW Jr, Chourey PS, Hannah, LC, Koch K (1996) Phosphorylation of serine-15 of maize leaf sucrose synthase. Occurrence in vivo and possible regulatory significance. Plant Physiol 112: 793–802.

Johnson KL, Cassin AM, Lonsdale A, Bacic A, Doblin MS, Schultz CJ (2017) Pipeline to identify hydroxyproline-rich glycoproteins. Plant Physiol 174: 886–903.

Kadam NN, Tamilselvan A, Lawas LMF, Quinones C, Bahuguna RN, Thomson MJ, Dingkuhn M, Muthurajan R, Struik PC, Yin X, et al (2017) Genetic control of plasticity in root morphology and anatomy of rice in Response to Water Deficit. Plant Physiol 174: 2302–2315

Kim H, Park M, Kim SJ, Hwang I (2005) Actin filaments play a critical role in
vacuolar trafficking at the Golgi complex in plant cells. Plant Cell 17: 888–902

Koch K (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr Opin Plant Biol 7: 235–246

Koroleva OA, Tomlinson ML, Leader D, Shaw P, Doonan JH (2005) High-throughput protein localization in Arabidopsis using Agrobacterium-mediated transient expression of GFP-ORF fusions. Plant J 41:162–174

Labeit S, Kolmerer B (1995) Titins: giant proteins in charge of muscle ultrastructure and elasticity. Science 270: 293–296

Li J, Henty-Ridilla JL, Staiger BH, Day B, Staiger CJ (2015) Capping protein integrates multiple MAMP signalling pathways to modulate actin dynamics during plant innate immunity. Nat Commun 6: 7206

Liang BM, Sharp RE, Baskin TI (1997) Regulation of growth anisotropy in well-watered and water-stressed maize roots. I. Spatial distribution of longitudinal, radial, and tangential expansion rates. Plant Physiol 115: 101–111

Ma K, Wang K (2002) Interaction of nebulin SH3 domain with titin PEVK and myopalladin: implications for the signaling and assembly role of titin and nebulin. FEBS Lett 532: 273–278

Marc J, Granger CL, Brincat J, Fisher DD, Kao Th, McCubbin AG, Cyr RJ (1998) A GFP-MAP4 reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. Plant Cell 10: 1927–1940

Panzner S, Dreier L, Hartmann E, Kostka S, Rapoport TA (1995) Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. Cell 181: 561–570

Paredez AR, Somerville CR, Ehrhardt DW (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312: 1491–1495
Real-Hohn A, Zancan P, Da Silva D, Martins ER, Salgado LT, Mermelstein CS, Gomes AM, Sola-Penna M (2010) Filamentous actin and its associated binding proteins are the stimulatory site for 6-phosphofructo-1-kinase association within the membrane of human erythrocytes. Biochimie 92: 538–544

Reiersen H, Rees AR (2001) The hunchback and its neighbours: proline as an environmental modulator. Trends Biochem Sci 26: 679–684

Rellán-Álvarez, Lobet G, Dinneny JR (2016) Environmental control of root system biology. Annu Rev Plant Biol 67: 619–642

Riedl J, Crevenna AH, Kessenbrock K, Yu JH, Neukirchen D, Bista M, Bradke F, Jenne D, Holak TA, Werb Z, et al (2008) Lifeact: a versatile marker to visualize F-actin. Nat Methods 5: 605–607

Ruff KM, Roberts S, Chilkoti A, Pappu RV (2018) Advances in understanding stimulus-responsive phase behavior of intrinsically disordered protein polymers. J Mol Biol 430: 4619–4635

Schindelin J, Arganda-Carreras I, Frise, E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, et al (2012) Fiji: an open-source platform for biological-image analysis. Nat Methods 9: 676–682

Schwartz MA, Ginsberg MH (2002) Networks and crosstalk: integrin signalling spreads. Nat Cell Biol 4: E65–68

Smith LG (2003) Cytoskeletal control of plant cell shape: getting the fine points. Curr Opin Plant Biol 6: 63–73

Smith LG, Oppenheimer DG (2005) Spatial control of cell expansion by the plant cytoskeleton. Annu Rev Cell Dev Biol 21: 271–295

Sugimoto K, Williamson RE, Wasteneys GO (2000) New techniques enable comparative analysis of microtubule orientation, wall texture, and growth rate in intact roots of Arabidopsis. Plant Physiol 124: 1493–1506
Sugimoto-Shirasu K, Carpita NC, McCann MC (2004) The cell wall: a sensory panel for signal transduction. In: Hussey PJ (ed) The Plant Cytoskeleton in Cell Differentiation and Development. Annual Plant Reviews 10: 176–203

Sun J, Loboda T, Sung SJ, Black CC (1992) Sucrose synthase in wild tomato, Lycopersicon chmielewskii, and tomato fruit sink strength. Plant Physiol 98: 1163–1169

Szymanski DB, Cosgrove DJ (2009) Dynamic coordination of cytoskeletal and cell wall systems during plant cell morphogenesis. Curr Biol 19: R800–811

Tompa P, Bánki P, Bokor M, Kamasa P, Kovács D, Lasanda G, Tompa K (2006) Protein–water and protein-buffer interactions in the aqueous solution of an intrinsically unstructured plant dehydrin: NMR intensity and DSC aspects. Biophys J 91: 2243–2249

Tseng IC, Hong CY, Yu SM, Ho TH (2013) Abscisic acid- and stress-induced highly proline-rich glycoproteins regulate root growth in rice. Plant Physiol 163: 118–134

Tzfira T, Tian GW, Lacroix B, Vyas S, Li J, Leitner-Dagan Y, Krichevsky A, Taylor T, Vainstein A, Citovsky V (2005) pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol Biol 57: 503–516

Uversky VN (2011) Intrinsically disordered proteins from A to Z. Int J Biochem Cell Biol 43: 1090–1103

Uversky VN (2016) Dancing Protein Clouds: The Strange Biology and Chaotic Physics of Intrinsically Disordered Proteins. J Biol Chem 291: 6681–6688

Wang K, Knipfer M, Huang QQ, van Heerden A, Hsu LC, Gutierrez G, Quian XL, Stedman H (1996) Human skeletal muscle nebulin sequence encodes a blueprint for thin filament architecture. Sequence motifs and affinity profiles of tandem repeats
and terminal SH3. J Biol Chem 271: 4304–4314

Wang S, Kurepa J, Hashimoto T, Smalle JA (2011) Salt stress-induced disassembly of Arabidopsis cortical microtubule arrays involves 26S proteasome-dependent degradation of SPIRAL1. Plant Cell 23: 3412–3427

Wienken CJ, Baaske P, Rothbauer U, Braun D, Duhr S (2010) Protein-binding assays in biological liquids using microscale thermophoresis. Nat Commun 1: 100

Winter H, Huber JL, Huber SC (1998) Identification of sucrose synthase as an actin-binding protein. FEBS Lett 430: 205–208

Winter, H., and Huber, S.C. (2000). Regulation of sucrose metabolism in higher plants: localization and regulation of activity of key enzymes. Crit Rev Biochem Mol Biol. 35: 253–289

Wulf E, Deboben A, Bautz FA, Faulstich H, Wieland T (1979). Fluorescent phallotoxin, a tool for the visualization of cellular actin. Proc Natl Acad Sci USA 76: 4498–4502

Xu K, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailey-Serres J, Ronald PC, Mackill DJ (2006) Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature 442: 705–708

Xue B, Dunbrack RL, Williams RW, Dunker AK, Uversky VN (2010) PONDR-FIT: a meta-predictor of intrinsically disordered amino acids. Biochim Biophys Acta 1804: 996–1010

Yang W, Ren S, Zhang X, Gao M, Ye S, Qi Y, Zheng Y, Wang J, Zeng L, Li Q, et al (2011) BENT UPPERMOST INTERNODE1 encodes the class II formin FH5 crucial for actin organization and rice development. Plant Cell 23: 661–680

Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2: 1565-1572
Figure 1. *In Vitro* Interactions of Recombinant OsRePRP2.1 (rOsRePRP2.1) and Actin/Tubulin.

(A) Segmental repeat organization of OsRePRP2.1. The diagram shows the full-length amino acid (aa) sequences analyzed by RADAR.
The repeat modules of OsRePRP2.1 are represented as blue and pink blocks and the non-repeat regions are white blocks. The percentage of the amino acid sequences are distinguished by colors of one-letter codes, red for 100%, green for >75%, blue for >67%, black for >50% and dash for non-consensus amino acids. (B) The binding affinity of rOsRePRP2.1 with actin/tubulin. *In vitro* binding assays were conducted with microscale thermophoresis (MST) and the binding affinity is represented as dissociation constant \((K_d)\) values. Asterisks indicate the binding affinity with synthetic peptides. (C-F) Binding isotherms for rOsRePRP2.1\(^{60-179}\) (C-D) and synthetic peptides OsRePRP2.1\(^{60-87}\) (E-F) to rabbit muscle actin (C, E) and porcine brain tubulin (D, F). Rabbit muscle actin and porcine brain tubulin were fluorescently labeled with AlexaFluor 647 and kept at a constant concentration of 1.2-1.5 nM. The binding of OsRePRP2.1 alone to actin and tubulin is represented as blue and green curves, respectively, and the binding of OsRePRP2.1 in the presence of AG is represented as pink-dashed curves. Closed circles indicate rOsRePRP2.1\(^{60-179}\) alone (C-D) and synthetic peptides OsRePRP2.1\(^{60-87}\) alone (E-F). Open triangles indicate rOsRePRP2.1\(^{60-179}\) (C-D) and OsRePRP2.1\(^{60-87}\) (E-F) in the presence of 250 \(\mu\)M arabinogalactan (AG). Closed squares indicate rOsRePRP2.1\(^{60-179}\) and synthetic peptides OsRePRP2.1\(^{60-87}\) in the presence of 25 \(\mu\)M porcine brain tubulin (C, E) or 57.5 \(\mu\)M rabbit muscle actin (D, F). Data are mean±SD of three technical repeats. G-actin and \(\alpha\beta\)-tubulin dimmers were used, and at least two independent batches of recombinant proteins or synthetic peptides were subjected to the binding assays.
Figure 2. Interactions between OsRePRP2 and Actin and Tubulin in Rice Root Protoplasts.

Five-day-old wild-type rice root protoplasts co-expressing LifeAct-GFP and OsRePRP2-DsRed (A-D), GFP-MBD and OsRePRP2-DsRed (E-H), LifeAct-GFP and GFP-MBD (I-L), DsRed (M-P), and DsRed channel (Q-T). The Pearson's R values are shown in the respective panels. The box plots in panel U compare the interactions between LifeAct-GFP vs RePRP2-DsRed, GFP-MBD vs RePRP2-DsRed, LifeAct-GFP vs DsRed, and GFP-MBD vs DsRed.
DsRed (I-L), and GFP-MBD and DsRed (M-P). GFP and DsRed signals are shown in green (A, E, I, M) and magenta (B, F, J, N); overlay of green and red channels are in (C, G, K, O), and the DIC channel indicating bright field images in (D, H, L, P). Five-day-old wild-type rice root protoplasts expressing LifeAct-GFP (Q-R) and GFP-MBD (S-T), GFP signals are shown in green (Q, S) while the respective control images in the DsRed channel are shown in (R, T). The Pearson's R correlation coefficients calculated by Pearson coefficient analysis are shown in a box plot (U). The Pearson's R coefficients for LifeAct-GFP and OsRePRP2-DsRed, GFP-MBD and OsRePRP2-DsRed, LifeAct-GFP and DsRed, and GFP-MBD and DsRed are shown in green, blue, magenta and black boxes, respectively (U). The line inside the box indicates the median, and the cross indicates the mean. Box edges are the 25th to 75th percentiles; whiskers indicate the range and an outlying data point is shown as a circle. Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Data are mean/median (Q1-Q3) for 15 protoplasts from three biological experiments. The Pearson's R correlation coefficients for each comparison are shown in (C, G, K, O). Bar = 2 μm.
Figure 3. Interactions between OsRePRP and Actin and Tubulin in Planta.

(A-B, E-G) Double immunogold-labeling transmission electron microscopy of root elongation zone cells of 3-day-old OsRePRP1-GFP (A-B) and OsRePRP2-GFP (E-G) rice. The smaller 12-nm gold particles represent the distribution of actin (A, E, G) and tubulin (B, F), and the larger 18-nm gold particles represent the distribution of OsRePRP1-GFP (A-B) and OsRePRP2-GFP (E-G). Arrows indicate co-localization of OsRePRP-GFP and actin (A, E, G) or tubulin (B, F). (C-D, H-I) The distances from each OsRePRP-GFP gold particle to the closest actin or tubulin gold particle were measured as described in Methods. The bars represent the distribution of inter-gold-particle-center distances between OsRePRP-GFP and actin or tubulin ranging from 0 nm to > 100 nm. Ten images were measured, and two biological replicates were performed. Bar = 100 nm in A-B, E-G.
Figure 4. OsRePRP Affects F-actin Organization.

(A-F) F-actin organization in the wild-type (WT), OsRePRP2-OX and OsRePRP-Ri root cells visualized by Alexa Fluor 488-phalloidin staining. The epidermal cells of the root elongation zones of 8-day-old seedlings (control; A-C) and 7-day-old seedlings treated with 2 μM ABA for 1 day (ABA; D-F) were observed. (G) Quantitative analysis of F-actin level in WT, OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) 8-day-old seedlings (control) and 7-day-old seedlings treated with 2 μM ABA for 1 day (ABA). Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Data are mean±SD fluorescence intensity of 30 images. More than six biological replicates were performed and at least three independent transgenic lines for each genotype were observed (OsRePRP2-OX #2, #10, #19, #24 and OsRePRP-Ri #5, #6, #7). Bar = 10 μm.
Figure 5. OsRePRP Affects F-actin Organization in Root Protoplasts.

The transient expression of LifeAct-GFP in 5-day-old rice root protoplasts from the wild type (A, D), OsRePRP2-OX (B, E) and OsRePRP-Ri (C, F). GFP signals are shown in green (A-C) and the DIC channel indicates bright field images in (D-F). Images in (A-C) represent the maximum intensity projection of 20 optical confocal images with identical settings. At least two independent transgenic lines for each genotype were observed (OsRePRP2-OX #2, #10, #24 and OsRePRP-Ri #5, #6, #7). Bar = 2 μm.
Figure 6. OsRePRP Affects Microtubule Organization.

(A-C) Micrographs of microtubule staining in WT (A), OsRePRP2-OX (B) and OsRePRP-Ri (C) root cells of elongation zones. (D-F) Micrographs of microtubule staining in WT (D), OsRePRP2-OX (E) and OsRePRP-Ri (F) root cells after 2 μM ABA treatment for 2 days. More than three biological replicates were performed and
three independent transgenic lines for each genotype were observed (OsRePRP2-OX #10, #19, #24 and OsRePRP-Ri #5, #6, #7). (G-L) The transient expression of GFP-MBD in 5-day-old rice root protoplasts from the WT (G, J), OsRePRP2-OX (H, K) and OsRePRP-Ri (I, L). GFP signals are shown in green (G-I) and the DIC channel indicates bright field images in (J-L). Four biological replicates were performed and three independent transgenic lines for each genotype were observed (OsRePRP2-OX #2, #10, #24 and OsRePRP-Ri #5, #6, #7). Bar = 2 μm.
Figure 7. OsRePRP Affects Non-cellulosic Polysaccharide Secretion and Cell-Wall Cellulose Microfibril Network.

(A-C) Localization of fucose alkyne (FucAl) incorporation in WT (A), OsRePRP2-OX (B) and OsRePRP-Ri (C) root elongation zone cells. Images were obtained with identical settings including laser power, pinhole, objective, zoom, and channel/filter wavelengths as described in Methods with no image contrast or brightness corrections. (D) Quantitative analysis of FucAl-associated fluorescence signal in the...
WT, OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) are shown in a box plot. The line inside the box indicates the median, and the cross indicates the mean. Box edges are the 25th to 75th percentiles; whiskers indicate the range. Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Data are mean/median (Q1-Q3) fluorescence intensity of 30 images. More than four biological replicates were performed and at least three independent transgenic lines for each genotype were observed (OsRePRP2-OX #2, #10, #19, #24 and OsRePRP-Ri #5, #6, #7). (E-J) Field-emission scanning electron microscopy images are oriented to show cell-wall cellulose microfibril alignment relative to the cell and the root long axis. (E-G) Micrographs of cell-wall cellulose microfibril network in WT (E), OsRePRP2-OX (F) and OsRePRP-Ri (G) root elongation zone cells under the control condition. (H-J) Micrographs of cell-wall microfibril network in WT (H), OsRePRP2-OX (I) and OsRePRP-Ri (J) root elongation zone cells after 2 μM ABA treatment for 4 days. Three biological replicates were performed and at least two independent transgenic lines for each genotype were observed (OsRePRP2-OX #10, #19, #24 and OsRePRP-Ri #5, #6). (K-L) Directionality histograms of cell wall cellulose microfibril network under the control condition (K) or after ABA treatment (L). Directionality analysis of WT (black line), OsRePRP2-OX (magenta line) and OsRePRP-Ri (blue line) involved using the Fiji package (Schindelin et al., 2012) as described in Methods. Data are mean frequency of fibers in specific orientation analyzed from 15 images. (M) Relative density of root segments in WT, OsRePRP2-OX and OsRePRP-Ri. Two-cm root-tip segments from 12-day-old seedlings (control) and 8-day-old seedlings treated with 2 μM ABA for 4 days (ABA) were cut and immersed in the distilled water. Six biological replicates were performed and three independent transgenic lines for each genotype were observed (OsRePRP2-OX #10, #19, #24 and OsRePRP-Ri #5, #6, #7). (N) Quantitative analysis of total root dry weight (mg) divided by mean root length (cm) per seedling. Whole roots were harvested from WT, OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) 14-day-old seedlings (control) and 8-day-old seedlings treated with 2 μM ABA for 6 days (ABA). Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Data are mean±SD of six biological repeats. Three independent transgenic lines for each genotype were measured (OsRePRP2-OX #2, #10, #24 and OsRePRP-Ri #5, #6, #7). Bar = 2 μm in (A-C), 100 nm in (E-J), 1 cm in (M).
Figure 8. Starch Accumulation in Rice Roots after PEG Treatment.

(A-B) Rice roots from 14-day-old seedlings of WT, OsRePRP2-OX and OsRePRP-Ri under control (A) or PEG (B) treatments stained with iodine. Squares in (A-B) represented the sectioning parts in (C-Q). Four biological replicates were performed and at least two independent transgenic plants for each genotype were observed (OsRePRP2-OX #2, #24 and OsRePRP-Ri #5, #6, #7). (C-Q) Cross and longitudinal root sections of WT (C, F, I, L, O), OsRePRP2-OX (D, G, J, M, P) and OsRePRP-Ri (E, H, K, N, Q) under control (C-K) or PEG (L-Q) treatments stained with iodine. Three biological replicates were performed and at least two independent transgenic lines for each genotype were observed (OsRePRP2-OX #10, #19, #24 and OsRePRP-Ri #5, #7). (R) Starch content measurements in the whole roots of WT, OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) under control or PEG treatments. Significant
differences are indicated with asterisks (P < 0.05, two-tailed Mann-Whitney U test). Data are mean±SD of six technical repeats. Three biological replicates were performed and two independent transgenic lines for each genotype were measured (OsRePRP2-OX #10, #24 and OsRePRP-Ri #5, #6). Bar = 1 cm in (A-B) and 50 µm in (C-Q).
Figure 9. Enzyme Activity of Sucrose Synthase (SUS) Was Changed in OsRePRP2-OX.

(A) Co-immunoprecipitation assays of GFP, OsRePRP1-GFP and OsRePRP2-GFP transgenic rice. Total protein was extracted from whole roots of 4-day-old seedlings...
treated with 2 μM ABA for 2 days. Western blot analysis involved anti-SUS antibodies. The arrow indicates 93-kDa SUS. Two biological replicates were performed. (B-C) Enzyme activity of sucrose synthase in the cleavage (B) and synthetic (C) directions. Total protein extract from 14-day-old whole roots of the wild type (WT), OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) was shown in box plots. The line inside the box indicates the median, and the cross indicates the mean. Box edges are the 25th to 75th percentiles; whiskers indicate the range. Significant differences are indicated with asterisks (P < 0.01, two-tailed Mann-Whitney U test). Data are mean/median (Q1-Q3) of nine repeats from three independent biological replicates. Five biological replicates were performed. (D) PhosTag western blot (WB) and WB analysis with anti-SUS antibodies. Total protein extract from 14-day-old whole roots of the wild type (WT), OsRePRP2-OX (OX) and OsRePRP-Ri (Ri) was assayed. Four biological replicates were performed.
al-Habori M (1995) Microcompartmentation, metabolic channelling and carbohydrate metabolism. Int J Biochem Cell Biol 27: 123–132

Anderson CT, Wallace IS, Somerville CR (2012) Metabolic click-labeling with a fucose analog reveals pectin delivery, architecture, and dynamics in Arabidopsis cell walls. Proc Natl Acad Sci USA 109: 1329–1334

Arendt ET, Rueden CT, Hiner MC, Wang S, Yuan M, Eliezeri KW (2017) Quantitating the cell: turning images into numbers with ImageJ. Wiley Interdiscip Rev Dev Biol 6: e260

Baba I, Takahashi Y (1956) Water and sand culture methods. In: Togari Y, Matsuo T, Hatamari M, Yamada N, Harada T, Suzuki N, editors. Experimental methods in crop science. Tokyo: Association of Agricultural Techniques p157–185

Balasubramanian R, Karve A, Kandasamy M, Meagher RB, Moore BD (2007) A role for F-actin in hexokinase-mediated glucose signaling. Plant Physiol 145: 1423–1434

Baluska F, Hlavacka A, Samaj J, Palme K, Robinson DG, Matoh T, McCurdy DW, Menzel D, Volkman D (2002) F-actin-dependent endocytosis of cell wall pectins in meristematic root cells. Insights from brefeldin A-induced compartments. Plant Physiol 130: 422–431

Baluska F, Samaj J, Wojtaszek P, Volkman D, Menzel D. (2003) Cytoskeleton-plasma membrane-cell wall continuum in plants. Emerging links revisited. Plant Physiol 133: 482–491

Bashline L, Li S, Gu Y (2014) Cell wall, cytoskeleton, and cell expansion in higher plants. Mol Plant 7: 586–600

Baskin TI (2005) Anisotropic expansion of the plant cell wall. Annu Rev Cell Dev Biol 21: 203–222

Baskin TI, Meekes HT, Liang BM, Sharp RE (1999) Regulation of growth anisotropy in well-watered and water-stressed maize roots. II. Role of cortical microtubules and cellulose microfibrils. Plant Physiol 119: 681–692

Bergersen LH, Storm-Mathisen J, Gundersen V (2008) Immunogold quantification of amino acids and proteins in complex subcellular compartments. Nat Protoc 3: 144–152

Boothby TC, Tapia H, Brozena AH, Piszkiewicz S, Smith AE, Giovannini I, Rebecchi L, Pielak GJ, Koshland D, Goldstein B (2017) Tardigrades Use Intrinsically Disordered Proteins to Survive Desiccation. Mol Cell 65: 975–984

Bray EA (1997) Plant responses to water deficit. Trends Plant Sci 2: 48–54

Buckley TN (2019) How do stomata respond to water status? New Phytol 224: 21–36

Candat A, Paszkiewicz G, Neveu M, Gautier R, Logan DC, Avelange-Macherel MH, Macherel D (2014) The ubiquitous distribution of late embryogenesis abundant proteins across cell compartments in Arabidopsis offers tailored protection against abiotic stress. Plant Cell 26: 3148–3166

Chuong SD, Good AG, Taylor GJ, Freeman MC, Moorhead GB, Muench DG (2004) Large-scale identification of tubulin-binding proteins provides insight on subcellular trafficking, metabolic channelling, and signaling in plant cells. Mol Cell Proteomics 3: 970–983
Covarrubias AA, Cuevas-Velazquez CL, Romero-Pérez P.S, Rendón-Luna DF, Chater CCC (2017) Structural disorder in plant proteins: where plasticity meets sessility. Cell Mol Life Sci 74: 3119–3147

Crowell EF, Bischoff V, Desprez T, Rolland A, Stierhof YD, Schumacher K, Gonneau M, Höfte H, Vernhettes S (2009) Pausing of Golgi bodies on microtubules regulates secretion of cellulose synthase complexes in Arabidopsis. Plant Cell 21: 1141–1154

Deng ZY, Liu LT, Li T, Yan S, Kuang BJ, Huang SJ, Yan CJ, Wang T (2015) OsKinesin-13A is an active microtubule depolymerase involved in glueme length regulation via affecting cell elongation. Sci Rep 25: 9457

Driouich A, Faye L, Staehelin LA (1993) The plant Golgi apparatus: a factory for complex polysaccharides and glycoproteins. Trends Biochem Sci 18: 210–214

Duncan KA, Huber SC (2007) Sucrose synthase oligomerization and F-actin association are regulated by sucrose concentration and phosphorylation. Plant Cell Physiol 48: 1612–1623

Era A, Tominaga M, Ebine K, Awa C, Saito C, Ishizaki K, Yamato KT, Kohchi T, Nakano A, Ueda T (2009) Application of Lifeact reveals F-actin dynamics in Arabidopsis thaliana and the liverwort, Marchantia polymorpha. Plant Cell Physiol 50: 1041–1048

Evrard A, Bargmann BO, Birnbaum KD, Tester M, Baumann U, Johnson AA (2012) Fluorescence-activated cell sorting for analysis of cell type-specific responses to salinity stress in Arabidopsis and rice. Methods Mol Biol 913: 265–276

Finkelstein RR, Gampala SS, Rock CD (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell 14 Suppl: S15–45

Fujita S, Pytel J, Hotta T, Kato T, Hamada T, Akamatsu R, Ishida Y, Kutsuna N, Hasezawa S, Nomura Y, et al (2013) An atypical tubulin kinase mediates stress-induced microtubule depolymerization in Arabidopsis. Curr Biol 23: 1969–1978

Fukao T, Xu K, Ronald PC, Bailey-Serres J (2006) A variable cluster of ethylene response factor-like genes regulates metabolic and developmental acclimation responses to submergence in rice. Plant Cell 18: 2021–2034

Garagounis C, Kostaki KI, Hawkins TJ, Cummins I, Fricker MD, Hussey PJ, Hetherington AM, Sweetlove LJ (2017) Microcompartmentation of cytosolic aldolase by interaction with the actin cytoskeleton in Arabidopsis. J Exp Bot 68: 885–898

Giarola V, Hou Q, Bartels D (2017) Angiosperm plant desiccation tolerance: hints from transcriptomics and genome sequencing. Trends Plant Sci 22: 705–717

Grossman M, Ben-Chetrit N, Zhuravlev A, Afik R, Bassat E, Solomonov I, Yarden Y, Sag I (2016) Tumor cell invasion can be blocked by modulators of collagen fibril alignment that control assembly of the extracellular matrix. Cancer Res 76: 4249–4258

Gutierrez-Cruz G, Van Heerden AH, Wang K (2001) Modular motif, structural folds and affinity profiles of the PEVK segment of human fetal skeletal muscle titin. J Biol Chem 276: 7442–7449

Hu H, Juvekar A, Lyssiotis CA, Lien EC, Albeck JG, Oh D, Varma G, Hung YP, Ullas S, Lauring J, et al (2016) Phosphoinositide 3-Kinase Regulates Glycolysis through Mobilization of Aldolase from the Actin Cytoskeleton. Cell 164: 433–446
Hu H, Xiong L (2014) Genetic engineering and breeding of drought-resistant crops. Annu Rev Plant Biol 65: 715–741
Huber SC, Huber JL, Liao PC, Gage DA, McMichael RW Jr, Chourey PS, Hannah, LC, Koch K (1996) Phosphorylation of serine-15 of maize leaf sucrose synthase. Occurrence in vivo and possible regulatory significance. Plant Physiol 112: 793–802.
Johnson KL, Cassin AM, Lonsdale A, Bacic A, Doblin MS, Schultz CJ (2017) Pipeline to identify hydroxyproline-rich glycoproteins. Plant Physiol 174: 886–903.
Kadam NN, Tamilselvan A, Lawas LMF, Quinones C, Bahuguna RN, Thomson MJ, Dingkuhn M, Muthurajan R, Struik PC, Yin X, et al (2017) Genetic control of plasticity in root morphology and anatomy of rice in Response to Water Deficit. Plant Physiol 174: 2302–2315
Kim H, Park M, Kim SJ, Hwang I (2005) Actin filaments play a critical role in vacuolar trafficking at the Golgi complex in plant cells. Plant Cell 17: 888–902
Koch K (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. Curr Opin Plant Biol 7: 235–246
Koroleva OA, Tomlinson ML, Leader D, Shaw P, Doonan JH (2005) High-throughput protein localization in Arabidopsis using Agrobacterium-mediated transient expression of GFP-ORF fusions. Plant J 41:162–174
Labeit S, Kolmerrer B (1995) Titins: giant proteins in charge of muscle ultrastructure and elasticity. Science 270: 293–296
Li J, Henty-Ridilla JL, Staiger BH, Day B, Staiger CJ (2015) Capping protein integrates multiple MAMP signalling pathways to modulate actin dynamics during plant innate immunity. Nat Commun 6: 7206
Liang BM, Sharp RE, Baskin TI (1997) Regulation of growth anisotropy in well-watered and water-stressed maize roots. I. Spatial distribution of longitudinal, radial, and tangential expansion rates. Plant Physiol 115: 101–111
Ma K, Wang K (2002) Interaction of nebulin SH3 domain with titin PEVK and myopalladin: implications for the signaling and assembly role of titin and nebulin. FEBS Lett 532: 273–278
Marc J, Granger CL, Brincat J, Fisher DD, Kao Th, McCubbin AG, Cyr RJ (1998) A GFP-MAP4 reporter gene for visualizing cortical microtubule rearrangements in living epidermal cells. Plant Cell 10: 1927–1940
Panzner S, Dreier L, Hartmann E, Kostka S, Rapoport TA (1995) Posttranslational protein transport in yeast reconstituted with a purified complex of Sec proteins and Kar2p. Cell 181: 561–570
Paredes AR, Somervillle CR, Ehrhardt DW (2006) Visualization of cellulose synthase demonstrates functional association with microtubules. Science 312: 1491–1495
Real-Hohn A, Zancan P, Da Silva D, Martins ER, Salgado LT, Mermelstein CS, Gomes AM, Sola-Penna M (2010) Filamentous actin and its associated binding proteins are the stimulatory site for 6-phosphofructo-1-kinase association within the membrane of human erythrocytes. Biochimie 92: 538–544
