OsSIZ2 exerts regulatory influences on the developmental responses and phosphate homeostasis in rice

Wenxia Pei1, Ajay Jain2, Yafei Sun1, Zhantian Zhang1, Hao Ai1, Xiuli Liu1, Huadun Wang1,3, Bing Feng1, Rui Sun1, Hongmin Zhou1, Guohua Xu1 & Shubin Sun1

OsSIZ1, a small ubiquitin-related modifier (SUMO) E3 ligase, exerts regulatory influences on the developmental responses and phosphate (Pi) homeostasis in rice (Oryza sativa). Whether paralogs OsSIZ1 and OsSIZ2 are functionally redundant or the latter regulates these traits independent of the former is not known. To determine this, in this study, OsSIZ2 was functionally characterized by employing reverse genetic approaches. Although the relative expression of OsSIZ2 was spatiotemporally regulated, it showed constitutive expression in root and leaf blade irrespective of Pi regime. Analysis of T-DNA insertion knockout (ossiz2) and RNAi-mediated knockdown (Ri1-3) mutants revealed positive influences on growth and developmental responses including yield-related traits. On the contrary, these mutants exhibited negative effects on the concentrations of Pi and total P in different tissues. The relative expression levels of some of the genes that are involved in Pi sensing and signaling cascades were differentially modulated in the mutants. Further, attenuation in the expression levels of OsSIZ2 in the roots of ossiz1 and relatively similar trend of the effects of the mutation in OsSIZ1 and OsSIZ2 on growth and development and total P concentration in different tissues suggested a prevalence of partial functional redundancy between these paralogs.

The current world population of 7.3 billion is expected to escalate to 9.7 billion by 2050 (www.un.org/en/development). For sustainably feeding such a large population, food production needs to be increased by ~70% (www.fao.org). Rice (Oryza sativa) is a staple for almost half of the world’s seven billion people of which ~90% is consumed in Asia alone (www.irri.org/rice-today). The world’s dietary energy supply is contributed maximum by rice (20%), followed by wheat (19%) and maize (5%) (www.research.cornell.edu). Rice is thus an ideal crop for global food security and its increased production and productivity is needed now more than ever.

The essential macronutrient phosphorus (P), a structural component of cellular macromolecules such as phospholipids and nucleic acids, plays a key role in energy-dependent metabolic pathways in plants1–2. Inorganic phosphate (Pi) is the preferred form of P taken up by plants of which 80% is immobile and not readily available in rhizospheres3. Pi deficiency is common in many of the rice ecosystems, particularly in acid upland soils with high soil P-fixation capacity and adversely affects root development, tillering and flowering4,5. Although soils are conventionally enriched with P fertilizer, its excessive usage is uneconomical for sustainable agriculture and also poses a threat to the environment. Therefore, it is imperative to develop plants with higher Pi use efficiency by biotechnological interventions6,7. In this context, it would be worthwhile to identify some of the molecular entities that influence the maintenance of Pi homeostasis. These molecular entities can potentially be engineered for generating ‘smart’ plants amenable for soils poor in Pi.

Plants have evolved an array of adaptive responses to circumvent the adverse effects of Pi deficiency8. Genome-wide transcriptome analysis of rice (Oryza sativa) seedlings grown under different Pi regime has facilitated in identifying spatiotemporal variations in transcript abundance of an array of a functionally diverse group of genes9–12. Current advances in RNA-Seq transcriptome analysis has expedited in identifying differently transcribed genes in Pi-deprived rice at single-base resolution13,14. Many of these genes are regulated by a repertoire of

1 State Key Laboratory of Crop Genetics and Germplasm Enhancement, Key Laboratory of Plant Nutrition and Fertilization in Low-Middle Reaches of the Yangtze River, Ministry of Agriculture, Nanjing Agricultural University, 210095, Nanjing, China. 2 Amity Centre of Nano Biotechnology and Plant Nutrition, Kant Kalwar, NH-11C, Jaipur, 303002, India. 3 Present address: Provincial Key Laboratory of Agrobiology, Jiangsu Academy of Agricultural Sciences, Nanjing, China. Correspondence and requests for materials should be addressed to S.S. (email: sunshubin@njau.edu.cn)
transcriptional and post-translational events. Post-translational modifications such as sumoylation and ubiquitration are critical in determining the activity of proteins, which then mediate complex regulatory processes that are important for the functioning of a cell.

Sumoylation involves the attachment of small ubiquitin-like modifier (SUMO) peptide to the target proteins by a specific enzymatic cascade, which alters their activities, localizations and/or abilities to interact with other proteins. In Arabidopsis, SUMO E3 ligase \textit{AtSIZ1} (At5g60410) plays diverse roles in the developmental processes and various biotic and abiotic stresses. In vitro, sumoylation of MYB transcription factor \textit{AtPHR1} by \textit{AtSIZ1} and exaggerated Pi deficiency responses of the mutant \textit{siz1} provided evidence towards its role in regulating both negatively and positively different Pi deficiency responses in Arabidopsis. \textit{AtSIZ1} has also been implicated in negatively regulating Pi deficiency-induced modulation of root architecture by exerting influence on auxin patterning. \textit{OsSIZ1} and \textit{OsSIZ2} are the homologs of \textit{AtSIZ1} in rice, which partially complemented the morphological phenotype of \textit{siz1} in Arabidopsis. This suggested a likely role of \textit{OsSIZ1} and/or \textit{OsSIZ2} in the maintenance of Pi homeostasis. This assumption gained some credibility from a study which demonstrated elevated uptake of Pi in creeping bentgrass by heterologous expression of \textit{OsSIZ1}. Furthermore, the mutation in \textit{OsSIZ1} triggered elevated concentration of Pi and altered expression levels of several genes involved in the maintenance of Pi homeostasis in \textit{ossiz1}. This raised a pertinent question whether paralogs \textit{OsSIZ1} and \textit{OsSIZ2} are functionally redundant or the latter exerts independent regulatory influences on the various developmental responses and/or the homeostasis of Pi.

Here, T-DNA insertion knockout mutation and RNAi-mediated suppression were employed to study the function of \textit{OsSIZ2}. The study revealed positive regulatory influences of \textit{OsSIZ2} on various agronomic traits. Whereas, its negative regulatory influences were evident on the concentrations of Pi and total P in different tissues and differential effects on several genes that play pivotal roles in sensing and signaling cascades governing Pi homeostasis. Further, comparative analysis of \textit{ossiz1} and \textit{ossiz2} mutants suggested a partial functional redundancy between \textit{OsSIZ1} and \textit{OsSIZ2}.

**Results**

**Expression of OsSIZ2 is regulated spatiotemporally but not induced during Pi deficiency.** To determine whether expression of OsSIZ2 is spatiotemporally regulated, qRT-PCR was employed for assessing its relative expression levels in different tissues collected from 6 to 16-week-old wild-type rice grown in a pot soil (Fig. 1a). Collected samples represented both the vegetative (6 and 9-week-old plants) and reproductive (12 and 16-week-old plants) growth phases. Throughout these growth phases, the relative expression levels of OsSIZ2 were significantly higher in the leaf blade compared with other tissues. There were also elevated expression levels of OsSIZ2 in the leaf blade during Pi deficiency (Fig. 1b).
levels of OsSIZ2 in the basal stem and leaf sheath of a 16-week-old plant compared with the corresponding tissues of 6 to 12-week-old plants. Expression of OsSIZ2 was also relatively higher in the husk compared with the root and node 1 at the grain-filling stage. Notably, the expression levels of OsSIZ2 in the root did not exhibit any significant increment throughout the vegetative and reproductive growth phases. The study revealed the spatiotemporal regulation of OsSIZ2. To determine the effects of Pi deficiency on the relative expression levels of OsSIZ2, wild-type seedlings were grown hydroponically under different Pi regime for 14 d (Fig. 1b). Pi deficiency did not exert any significant influence on the relative expression levels of this gene in the root and leaf blade.

**Generation of OsSIZ2 knockout and knockdown mutants for functional characterization.** OsSIZ1, a paralog of OsSIZ2, has been shown to exert regulatory influences on different Pi deficiency responses22. Extensive concerted evolution of rice paralogs has led them on to a path of regaining independence25. Therefore, elucidation of the level of functional redundancy and divergence in the gene family in rice that plays a pivotal role in the maintenance of Pi homeostasis has often been an attractive paradigm. By employing phylogenetic and mutation analysis, functional redundancy and some degree of functional diversification were observed among four orthologs of *Arabidopsis thaliana* PHOSPHATE RESPONSE1 (PHR1) in rice (OsPHR1–4)26–28. Therefore, to determine whether OsSIZ2 is functionally redundant with OsSIZ1 and/or exerts distinctive influences on various morphophysiological and molecular responses governing Pi homeostasis, it was functionally characterized. Since reverse genetics is a potent technique for functional genomics in rice29,30, it was used for generating T-DNA insertion knockout (ossiz2 in Dongjin [DJ] background) and RNA interference (RNAi)-mediated knockdown (Ri1-3 in Nipponbare [NB] background) mutants (Supplementary Fig. S1). The insertion of the T-DNA was downstream of the OsSIZ2 translational start site and in the exon 5 (Supplemental Fig. S1a). Two-round PCR was employed for screening randomly selected 11 putative homozygous *ossiz2* knockout mutants by using (i) T-DNA right border-specific primer P and OsSIZ2-specific primer R1 and (ii) OsSIZ2-specific primers (F1 and R1) flanking the T-DNA insertion site (Supplemental Fig. S1a,b). The analysis revealed that ~55% of them (lines 3 to 8 marked with red boxes) are homozygous and are hereafter referred to as ossiz2 (Supplemental Fig. S1b). Semi-quantitative RT-PCR was carried out by using F2 and R2 primers for validating the lack of OsSIZ2 transcripts in ossiz2 mutants (lines 3, 5, 7 and 8; Supplemental Fig. S1c). Further, qRT-PCR was employed by using OsSIZ2-specific primers for determining the relative expression levels of OsSIZ2 in wild-types (NB and DJ) and the mutants (Ri1-3 and ossiz2) (Supplemental Fig. S1d). The relative expression levels of OsSIZ2 were significantly reduced (~35–58%) in Ri1-3 and undetected in ossiz2 compared with their corresponding wild-type. The results confirmed that Ri1-3 and ossiz2 are knockout and knockdown mutants, respectively of OsSIZ2. Southern blot analysis confirmed a single T-DNA insertion in ossiz2 (Supplemental Fig. S1e).

**Mutation in OsSIZ2 affects the growth and development.** The phenotypic traits of knockdown (Ri1-3) and knockout (ossiz2) OsSIZ2 mutants were compared with their corresponding wild-type and also with OsSIZ1 mutant (ossiz1) during vegetative and reproductive growth phases (Fig. 2). OsSIZ2 mutant (Ri1-3 and ossiz2) seedlings grown hydroponically in a +P medium for 4 weeks revealed retarded vegetative growth compared with their corresponding wild-type and their responses were analogous to ossiz1 (Fig. 2a). Similar attenuating effects were also explicit on the growth responses of these mutants (Ri1-3, ossiz1 and ossiz2) compared with their respective wild-type when grown to maturity (20-week-old) in a pot soil (Fig. 2b). The inhibitory effects of the mutation in OsSIZ1 and OsSIZ2 were also apparent in the development of the panicle (Fig. 2c). Further, effects of the mutation in OsSIZ1 and OsSIZ2 on various morphometric traits were compared with their respective wild-type (Fig. 2d–j). Ri1-3 showed significant reductions in biomass, root length, plant height, panicle length and weight of 1000 grains compared with NB (Fig. 2d,e,g,h and j). The number of primary rachis branches was significantly lower in R1 and 2 (but not in Ri3) compared with NB (Fig. 2i). However, root/shoot ratio of Ri1-3 was comparable with NB (Fig. 2f). The ossiz1 and ossiz2 mutants also exhibited the reductions in biomass, root length, plant height, the number of primary rachis branches and weight of 1000 grains compared with DJ (Fig. 2d,e,g,i and j). Compared with OsSIZ2, the effects of the mutation in OsSIZ1 were relatively more aggravated on some of these morphometric traits (Fig. 2e,g,i and j). This trend was more explicit with respect to panicle length, which was significantly lower in ossiz1 compared with DJ and ossiz2 (Fig. 2h). The present study thus suggested a pivotal role of OsSIZ2 in exerting positive regulatory influences on the developmental responses of different morphometric traits during vegetative and reproductive growth phases. Comparative analyses of these traits in ossiz2 with ossiz1 also revealed some degree of functional redundancy between OsSIZ2 and OsSIZ1.

**Mutation in OsSIZ2 affects uptake and mobilization of Pi during vegetative growth.** Since OsSIZ1 has been shown to play a role in the maintenance of Pi homeostasis23, it raised a pertinent question about a likely involvement of OsSIZ2. To investigate this, seedlings (3-4-d-old) of the wild-type (NB) and Ri1-3 were grown hydroponically for three weeks in +P and −P media and their root and leaf blade were assayed for Pi concentration (Fig. 3). Under +P condition, there were significant increases in the concentration of Pi in the root (~34–48%) and leaf blade (~36–65%) of Ri1-3 compared with the wild-type. Under −P condition, the concentration of Pi significantly increased in the root (~29–51%) and decreased in the leaf blade (~10–15%) of Ri1-3 compared with the wild-type. It was assumed that higher Pi concentration in the root (+P and −P) and leaf blade (+P) of Ri1-3 mutants could be due to elevated Pi acquisition by their roots. To investigate this, uptake rate (root) and distribution (shoot/root) of 32Pi was compared between the wild-type (NB) and the mutants (Ri1 and 2) under +P and −P conditions (Fig. 4). Although under +P condition, there was significantly higher (~36–47%) 32Pi uptake rate by roots of Ri1 and 2 compared with the wild-type, the corresponding values were significantly lower (~8–11%) under −P condition (Fig. 4a). The effects of different Pi regime were also evident on the 32Pi distribution (shoot/root) with the value being comparable between the wild-type and the mutants under +P condition, while it was significantly lower (~24–27%) in the mutants compared with the wild-type under
In an earlier study, the efficacy of Pi mobilization from the root to shoot was positively correlated with the Pi concentration in xylem sap. Therefore, Pi concentration in the xylem sap was assayed in the culms of wild-types (NB and DJ) and the mutants (Ri1-3 and ossiz2) grown in a pot soil up to the grain-filling stage (Fig. 5). Pi concentration in the xylem sap was significantly higher in Ri1 (~21–83%) and ossiz2 (~35%) compared with their corresponding wild-type.

Mutation in OsSIZ2 triggered elevated total P concentration in different tissues at grain-harvest stage. Total P concentration was assayed in different tissues of the wild-types (NB and DJ) and the mutants (Ri1-3, ossiz1 and ossiz2) grown in a pot soil up to the grain-harvest stage (Fig. 6). Total P concentration was significantly higher in Ri1 (~72% in leaf blade, ~3-fold in leaf sheath, ~44% in culm, ~27% in panicle axis and ~24% in brown rice) compared with NB. Total P concentration of Ri2 and 3 in leaf blade, leaf sheath and brown rice was relatively lower than Ri1, but significantly higher compared with NB. Whereas, total P concentration of Ri2
Figure 3. RNAi-mediated silencing of OsSIZ2 affects Pi concentration under different Pi regimes. Seedlings (3-d-old) of NB and RNAi plants (Ri1-3) in NB background were grown hydroponically in +P and −P media for three weeks and their root and leaf blade were assayed for Pi concentration. Values are means ± SE (n = 4) and asterisks indicate that the values were significantly (P < 0.05) different in the mutants compared with the WT. FW, Fresh weight.

Figure 4. RNAi-mediated silencing of OsSIZ2 affects uptake rate and distribution of 32Pi under different Pi regime. Rice seedlings (10-d-old) of NB and Ri1-2 were grown hydroponically in +P and −P media for 7 d. Seedlings were then transferred to +P and −P media labeled with 32Pi for 3 h. Roots and shoots of the labeled seedlings were separated. (a) Root was assayed for 32Pi uptake rate. (b) Root and shoot were assayed to determine the distribution of 32Pi between them. Values are means ± SE (n = 4) and asterisks indicate that the values were significantly (P < 0.05) different in the mutants compared with the WT.
and 3 in culm and panicle axis was comparable with NB. The data suggested that among the three OsSIZ2-RNAi transgenic lines, Ri1 was more robust in augmenting the total P concentration in different tissues. This accentuated response of Ri1 was consistent with its higher 32Pi uptake rate and Pi concentration in the xylem compared with Ri2 and/or 3 under +P condition (Figs 4a and 5). Although a similar trend of significantly elevated total P concentration was also observed in these tissues of ossiz1 and ossiz2 compared with DJ, the effect was more pronounced in ossiz1 than ossiz2.

**Mutation in OsSIZ2 differentially affects the relative expression levels of a subset of genes involved in the maintenance of Pi homeostasis.** Several genes involved in the sensing and signaling cascades that govern Pi homeostasis have been functionally characterized in rice32. Therefore, qRT-PCR was employed for assaying the relative expression levels of some of these genes in the roots of the wild-types (NB and DJ) and the mutants (Ri1 and ossiz2) grown hydroponically under +P and −P conditions for 14 d (Fig. 7). Biomass and root/shoot ratio of the mutants (Ri1-3) and were significantly (P < 0.05) attenuated compared with the wild-type under −P condition (data not shown). Under +P condition, the relative expression levels of OsPT1 and OsPHO1;2 in Ri1 and ossiz2 were significantly higher, while that of OsPHR2 and OsPAP10a were attenuated in ossiz2 compared with their respective wild-type. Under −P condition, the relative expression levels of these genes varied in the mutants ranging from suppression (OsPHR2, OsmiR399j and OsPAP10a) and induction (OsPT8 and OsPHO1;2) in both Ri1 and ossiz2, suppression only in Ri1 (OsiIPS1 and OsPT2) and ossiz2 (OsSQD2) and no effect on either of these mutants (OsPT1) compared with their corresponding wild-type. Overall, the relative expression analysis of Ri1-3 and ossiz2 provided evidence towards the differential regulatory influence of OsSIZ2 on a subset of molecular entities that governs Pi homeostasis in rice.

**OsSIZ1 exerts regulatory influence on OsSIZ1 in roots.** The relative expression levels of OsSIZ1 and OsSIZ2 were assayed in the leaf blade and root of the wild-type, ossiz1 and ossiz2 seedlings grown hydroponically under +P and −P conditions for 14 d (Fig. 8). As anticipated, there were barely any detectable expression levels of OsSIZ1 and OsSIZ2 in the leaf blade and root of ossiz1 and ossiz2, respectively under different Pi regime. In ossiz2, the relative expression levels of OsSIZ1 were higher in +P leaf blade, lower in +P root and were
unaffected in Pi-deprived leaf blade and root of ossiz2 as compared with the wild-type. Whereas in ossiz1, the relative expression levels of OsSIZ2 were attenuated in root and remained unaffected in leaf blade as compared with the wild-type under different Pi regime. The result provided some evidences towards the regulatory influence of OsSIZ1 on OsSIZ2 in roots of the seedlings grown under different Pi regime.

Discussion

Post-translational modifications (PTMs) are critical in determining the activity of proteins, which then mediate complex regulatory processes that are important for the functioning of a cell. These PTMs include processes such as phosphorylation, sumoylation, ubiquitination, N-terminal acetylation, carbonylation etc. Of these, sumoylation conjugates Small Ubiquitin-related MOdifier (SUMO) to substrate proteins through reversible post-translational modifications. It is implicated in transcriptional activation, degradation and localization of proteins, protein-protein interactions and is highly regulated by the environment. SUMO conjugation (Sumoylation) to the target proteins involves the sequential actions of activation (E1), conjugation (E2) and ligation (E3). There is one member of the Protein Inhibitor of Activated STAT (PIAS) group of SUMO E3 ligases (SIZs) in Arabidopsis (AtSIZ1) and two members in rice (OsSIZ1 and OsSIZ2). OsSIZ2 showed ubiquitous expression in different vegetative (6 to 9-week-old) and reproductive (12 to 16-week-old) tissues with a relatively higher level in the leaf blade during growth in a pot soil (Fig. 1a). Further, there were increments in the expression of OsSIZ2 in different tissues as the development progressed from the vegetative to reproductive phase of the life cycle (Fig. 1a). This suggested potential roles of OsSIZ2 in growth and development throughout the life cycle in rice.

OsSIZ1 also showed constitutive expression in different tissues with the expression being strongest in the leaves. OsSIZ1 and OsSIZ2 exhibit SUMO E3 ligase activity and are localized to the nucleus.

Reverse genetic approaches of T-DNA insertion-mediated knockout and/or RNA interference (RNAi)-mediated knockdown has expedited the process of functional genomics of rice, which is the first sequenced crop genome. The reverse genetic approach was used for the functional characterization of OsSIZ1 and OsSIZ2. Therefore, to functionally characterize the role of OsSIZ2, knockout (ossiz2) and knockdown (Ri1-3) mutants were generated (Supplemental Fig. S1). The locations of T-DNA insertions are preferred in the order of exon, intron, 5′-untranslated region and the promoter for effectively knocking out the function of the gene. Consistent with this notion, the insertion of the T-DNA was found to be in the exon 5 of OsSIZ2 (Supplemental Fig. S1a), which suggested its potential efficacy of knocking out the function of this gene. This was confirmed by qRT-PCR analysis, which revealed no transcripts of OsSIZ2 in ossiz2 mutant (Supplemental Fig. S1d). This was consistent with a review of about 1000 research published papers, which revealed that T-DNA insertion in exon was effective in generating a gene knock out ~90% of the time. Further, Southern blot analysis validated a single T-DNA insertion in ossiz2 (Supplemental Fig. S1e). In addition, the expression of OsSIZ2 was significantly reduced (~35–58%) in Ri1-3 (Supplemental Fig. S1d). The mutants (ossiz2 and Ri1-3) thus provided valuable genetic tools to determine the function of OsSIZ2.

To determine the effects of the mutation in OsSIZ2 on vegetative growth phase, seedling (3-d-old) of the wild-types (NB and DJ) and the mutants (Ri1-3, ossiz1 and 2) were grown in a pot soil for 20 weeks (grain-harvest stage). Data are presented for total P concentration in different tissues. Values are means ± SE (n = 5) and asterisks indicate that the values were significantly (P < 0.05) higher in the mutants compared with their corresponding WT. DW, Dry weight.
been implicated in the remodelling of root architecture by regulating auxin patterning\textsuperscript{19}. Genetics and functional genomics have expedited in the identification of several genes that play key roles in controlling root development in rice\textsuperscript{44}. It would be interesting to know if any of these genes involved in root development could potentially be

**Figure 7.** Mutation in OsSIZ2 exerts differential effects on the relative expression levels of the genes involved in Pi homeostasis. WTs (NB and DJ) and the mutants (Ri1 and ossiz2) were grown hydroponically under +P and −P conditions as described in the legend to Fig. 1b. Root tissues were subjected to qRT-PCR analysis for determining the relative expression levels of functionally different genes implicated in the maintenance of Pi homeostasis. OsActin was used as an internal control. Values are means ± SD (n = 3). Asterisks indicate that the values differ significantly (P < 0.05) in the mutants compared with their corresponding WT.
On the contrary, exerted positive regulatory influences on the uptake and distribution of 32Pi under OsSIZ2, similar negative regulatory influence of OsPT1. OsSIZ2 was also evident on the relative expression levels of transporters in the roots of ossiz1 in leaf blade and root of these seedlings. OsSIZ2 in the leaf blade and root of OsPT1 under different Pi regime. WT (D) and the mutants (ossiz1 and 2) were grown hydroponically under +P and −P conditions as described in the legend to Fig. 1b. qRT-PCR was employed for determining the relative expression levels of OsSIZ1 and OsSIZ2 in the leaf blade and root of these seedlings. OsActin was used as an internal control. Values are means ± SD (n = 3). Asterisks indicate that the values were significantly (P < 0.05) different in the mutants compared with the WT.

sumoylated by OsSIZ1 and/or OsSIZ2 and thus warrants further detailed studies. To further determine the effects of the mutation in OsSIZ2 on any of the reproductive traits, wild-types and the mutants (ossiz2 and Ri1-3) were grown in a pot soil for 20 weeks (Fig. 2b,c,g–j). These mutants showed significant reductions in plant height, the number of primary rachis branches and weight of 1000 grains compared with the wild-types. This clearly suggested broad-spectrum positive regulatory influences of OsSIZ2 on growth and development during vegetative and reproductive growth phases. OsSIZ1 has been also been implicated in regulating several traits representing reproductive growth phase. Since traits governing growth and development are polygenic, identifying sumoylated proteins in vivo can be a daunting task. In this context, an integrative analysis of known and putative SUMO substrates could provide a rich repository of SUMO-regulated events. Although studies on mining SUMO targets revealed that majority of them were transcription factors and genes involved in chromatin-related processes and/or RNA/DNA-dependent processes, some of them were not nucleus-localized. A novel proteomic approach involving 2-D liquid chromatography, immunodetection and mass spectroscopy analyses have also been demonstrated for the identification of novel SUMO targets. Overall, these integrative approaches can provide valuable insights into the mechanistic details of the sumoylated-mediated regulation of growth and development in rice.

Now there are growing evidence to implicate sumoylation in the maintenance of nutrient homeostasis. For instance in Arabidopsis, sumoylation plays key roles in Pi starvation responses, tolerance to excess copper and stimulation of nitrate reductase activity. Recently in rice, OsSIZ1 has also been shown to exert regulatory influences on several traits governing homeostasis of Pi and N. OsSIZ2 was not induced in Pi-deprived root and leaf blade (Fig. 1h). Non-responsiveness of OsSIZ2 to Pi deficiency was consistent with that of OsSIZ1. This suggested that sumoylation may be responding to different Pi regime by modification of proteins involved in the maintenance of Pi homeostasis downstream of OsSIZ1 and/or OsSIZ2. Furthermore, several genes that play a key role in the maintenance of Pi homeostasis such as transcription factor OsPHR1 and OsPHR2, Pi transporter OsPT1 and OsPT8, show constitutive expression irrespective of Pi regime. Elevated concentrations of Pi and total P in different tissues, and higher 32Pi uptake rate in the mutants (Ri1-3 and/or ossiz2) under +P condition (Figs 3–6) suggested negative regulatory influences of OsSIZ2 on physiological traits governing Pi homeostasis. A similar negative regulatory influence of OsSIZ2 was also evident on the relative expression levels of OsPT1 under +P condition, OsPT8 under −P condition and OsPHO1;2 under both +P and −P conditions (Fig. 7). On the contrary, OsSIZ2 exerted positive regulatory influences on the uptake and distribution of 32Pi under −P condition and the relative expression levels of OsPHR2, OsmiR399a, OsIPS1, OsPT2, OSPAP10 and OsSQD2 (Figs 4 and 7). This suggested that OsSIZ2 acts both positively and negatively on different molecular entities that govern Pi homeostasis. OsSIZ1 and SIZ1 in Arabidopsis also exhibited differential regulatory influences on the genes involved in Pi homeostasis. Attenuation in the relative expression of OsSIZ1 in +P root of ossiz2 and that of OsSIZ2 in −P root of ossiz1 (Fig. 8) suggested reciprocal influences on each other. Interestingly, a comparative analysis of the effects of the mutation on OsSIZ1 and OsSIZ2 (Fig. 7) on the relative expression levels of Pi transporters in the roots of ossiz1 and ossiz2 mutants revealed their positive (OsPT2) and negative (OsPT1 and OsPT8) regulatory influences. This suggested a prevalence of functional redundancy between these two paralogs. It is not surprising because members of the gene families in rice genome have a high probability (~51%) of being functionally redundant. But notably, the negative regulatory influence of OsSIZ1 on OsPT1 was under −P.
condition\textsuperscript{22}, whereas that of OsSIZ2 on this Pi transporter was under +P condition (Fig. 7). This indicated at least some degree of functional independence between these two genes.

Further, to get a better insight into the relative effects of the mutation in OsSIZ1 and OsSIZ2 on different agro- nomic traits, the wild-type (DJ) and the mutants (ossiz1 and ossiz2) were grown hydroponically in +P medium (4 weeks) and in a pot soil (20 weeks) for documenting the effects on the vegetative and reproductive traits (Fig. 2). Although during vegetative growth phase the reduction in biomass in both these mutants was comparable, the attenuating effect on root length was more robust in ossiz1 compared with ossiz2 (Fig. 2a,d,e). Relatively more aggravated effects of ossiz1 on the reproductive traits compared with ossiz2 were also evident (Fig. 2b,c,g–j). The results suggested a relatively dominant positive regulatory effect of OsSIZ1 compared with OsSIZ2 during vegetative and reproductive growth phases of rice. An earlier study has also shown that the expression of OsSIZ1 was ~2-fold higher than OsSIZ2 in different vegetative and reproductive tissues\textsuperscript{55}. Similarly, the negative regulatory effects of OsSIZ1 were more pronounced than OsSIZ2 on the total P concentration in different tissues (Fig. 6). Together the results suggested dominant regulatory effects of OsSIZ1 compared with OsSIZ2 on the responses related to growth and development and maintenance of Pi homeostasis.

Materials and Methods

Plant materials and growth conditions. Rice (\textit{Oryza sativa} L. ssp \textit{japonica}) wild-type (Nipponbare [NB] and Dongjin [DJ]), T-DNA insertion mutants (\textit{ossiz1} and \textit{2}) and OsSIZ2 RNAi plants (Ri1-3) in DJ and NB background, respectively were used in this study. Seeds were surface-sterilized as described\textsuperscript{22} and germinated on half-strength Murashige and Skoog (MS) medium in dark at 25 °C for 3 d. Seedlings with their radicle length in the range of 2–3 cm were transferred to a hydroponic set up containing +P medium and maintained in a growth room (16-h-light [30 °C]/8-h-dark [22 °C] and relative humidity ~70%) for 7 d. + P medium comprised KH\textsubscript{2}PO\textsubscript{4} (0.3 mM), NH\textsubscript{4}NO\textsubscript{3} (1.25 mM), CaCl\textsubscript{2} (1 mM), MgSO\textsubscript{4} (1 mM), Na\textsubscript{2}SiO\textsubscript{3} (0.5 mM), K\textsubscript{2}SO\textsubscript{4} (0.35 mM), K\textsubscript{2}HPO\textsubscript{4} (0.3 mM), Fe-EDTA (20 µM), H\textsubscript{2}BO\textsubscript{3} (20 µM), MnCl\textsubscript{2} (9 µM), ZnSO\textsubscript{4} (0.77 µM), Na\textsubscript{2}MoO\textsubscript{4} (0.39 µM) and CuSO\textsubscript{4} (0.32 µM) with pH adjusted to 5.5. Seedlings were then transferred to a hydroponic set up containing +P and –P (0 mM KH\textsubscript{2}PO\textsubscript{4}) media for 14 d. The nutrient media in the hydroponic set up were replaced every 3 d during the course of the experiment. Soil collected from the experimental farm at Nanjing Agricultural University was used for the pot experiments as described\textsuperscript{22}.

Identification of \textit{ossiz2} mutant. OsSIZ2 mutant line PFG_3A-13223.R with T-DNA from pGA2715 vector in DJ background was obtained from RiceGE (http://signal.salk.edu/RiceGE). Two-round PCR was performed for screening the homozygous mutants. The first-round PCR was carried out with T-DNA specific primer (P) and OsSIZ2-specific primers (R1) for identifying the mutants with T-DNA insertion. Subsequently, second-round PCR was performed with the primers (F1 and R1) flanking the T-DNA insertion site for identifying homozygous mutants. \textit{OsActin} was used as the reference gene. A list of primers used is given in Supplementary Table S1.

Generation of OsSIZ2-RNAi transgenics. To generate RNAi construct, OsSIZ2 coding sequence-specific 479 bp fragment (1050–1529 bp downstream of the ATG start codon defined as 1) was amplified by PCR. To facilitate subsequent cloning of the PCR product into the binary vector pTCK303, BamHI and KpnI sites were incorporated into the 5′ end of both forward primer AS-F (5′-GGATCCTTAAGACGCCACCTGTTC-3′) and reverse primer AS-R (5′-GTGGTACCGAGGCAGATAATGCTGACAG-3′). The purified PCR product was ligated to the TA cloning pMD18-T vector (TaKaRa) and positive clones were selected. The PCR-amplified fragment and pTCK303 were digested with BamHI and KpnI and cloned in the sense orientation as described\textsuperscript{56}. OsSIZ2-specific 479 bp fragment was then amplified using forward primer S-F (5′-ATGGTGTCGAGCTGAGATCTGACAG-3′) and reverse primer S-R (5′-GTGGTACCGAGGCAGATAATGCTGACAG-3′) with SacI and SpeI sites incorporated into their 5′ end. The PCR-amplified fragment was cloned in the antisense orientation in pTCK303 using a similar strategy. The presence of the two inserts in the desired orientation in the plasmid was confirmed by sequencing. The plasmid was transferred to \textit{Agrobacterium tumefaciens} strain EHA105 by electroporation and then transformed into embryonic calli (induced on N6 medium) of NB as described\textsuperscript{37}. Transgenic plants were selected on a medium containing hygromycin (50 mg L\textsuperscript{-1}) transferred to the soil and grown to maturity. Three independently generated OsSIZ2-RNAi transgenic lines (Ri1-3) were selected. qRT-PCR was used for validating the knockdown of the relative expression levels of OsSIZ2 in Ri1-Ri3 compared with the wild-type (Supplemental Fig. S1d).

Semi-quantitative RT-PCR and qRT-PCR analyses. Total RNAs from various tissues of the wild-types (NB and DJ) and the mutants (\textit{ossiz1} and \textit{2} and Ri1-3) isolated using Trizol reagent (Invitrogen). Semi-quantitative RT-PCR was carried out by using gene-specific primers for OsSIZ2. The PCR products were analyzed on agarose gel (1%, w/v) and images were captured with a CCD camera. For qRT-PCR, total RNA (~2µg) was treated with RNase-free DNase I and reverse transcribed using SuperScript III first-strand synthesis kit (Invitrogen). qRT-PCR analysis was performed on a StepOnePlus Real-Time PCR System (Applied Biosystem) using SYBR green master mix (Vazyme) and gene-specific primers. The relative expression levels of the genes were computed by 2\textsuperscript{ΔΔCt} method of relative quantification\textsuperscript{58}. \textit{OsActin} was used as a control for both semi-quantitative RT-PCR and qRT-PCR analyses. A list of primers used is given in Supplementary Table S1.

Southern blot analysis. The copy number in \textit{ossiz2} was determined by Southern blot analysis. The genomic DNA (80–100 µg) was digested overnight with HindIII and BglII at 37°C, separated on agarose gel (1%, w/v) and blotted onto a nylon membrane (Hybond N+, Amersham). The membrane was hybridized overnight in a hybridization solution as described\textsuperscript{59} containing DIG-labeled hygromycin phosphotransferase (25 ng ml\textsuperscript{-1}) probe. The membrane was washed twice with a solution containing 1xSSC and SDS (0.1%, w/v) for 15 min at 65°C. The washed membrane was analyzed with ScanMaker S260 (Microtek).
Quantification of Pi. Wild-type (NB) and the mutants (Ri1-3) were grown hydroponically in +P and −P media for three weeks and their root and leaf blade were harvested. For collection of the xylem sap, the wild-types (NB and DJ) and the mutants (Ri1-3 and osisz2) were grown in a pot soil for 16 weeks (grain-filling stage). An incision was made on their stem at 4 cm above the soil surface. The cut surface was rinsed with deionized water and blotted dry. A cotton wool pad was placed on the cut surface for 12 h to facilitate the absorption of xylem sap. Pi concentration in the tissues (root and leaf blade) and xylem sap (extracted from the cotton wool) was assayed as described.

Quantification of total P. Wild-types (NB and DJ) and the mutants (Ri1-3, osisz1 and 2) were grown in a pot soil for 20 weeks (grain-harvest stage) and different tissues (leaf blade, leaf sheath, culm, panicle axis and brown rice) were harvested. Dry tissues (~50 mg) were digested with H$_2$SO$_4$ (5 ml) in glass tubes overnight at room temperature. The tubes were then heated to 280 °C and H$_2$O$_2$ (6–8 drops) were added at an interval of 10 min until the solution turned colourless. The digested sample was diluted to 100 ml with deionized water and total P concentration was assayed as described.

Assay for the uptake and distribution of $^{32}$Pi. Rice seedlings (10-d-old) of the wild-type (NB) and the mutants (Ri1,2) were grown in +P and −P media for 7 d. The seedlings were then transferred to the nutrient solution supplemented with $^{32}$Pi (8 µCi, Perkin-Elmer) and grown for 3 h. To remove the apoplastic $^{32}$Pi, roots were washed thrice for 5 min each with ice-cold desorption solution (2 mM MES, 0.5 mM CaCl$_2$ and 0.1 mM NaH$_2$PO$_4$, pH 5.5). Seedlings were blotted-dry, root and shoot were separated and their fresh weights were documented. Samples were digested with HClO$_4$ (3 ml) and H$_2$O$_2$ (1 ml) at 25 °C for about 2 d with intermittent shaking till the solution turned colourless. Digestion mixture (300µl) was added to the scintillation cocktail (3.5 ml) and incubated for 4h with vigorous shaking at room temperature. $^{32}$Pi radioactivity was determined in both the root and shoot by using a liquid scintillation counter (Tri-Carb 2100 TR, Perkin Elmer). $^{32}$Pi counts in the root were used for determining the uptake rate, while that in the root and shoot were used for computing the distribution (shoot/root).

Statistical analysis. Data were collected from 2–3 independent biological experiments and analyzed for significant differences using IBM SPSS Statistics 20 program.

References
1. Marschner, H. In Mineral nutrition of higher plants, Second Edition. Academic Press, London (ed. Marschner, P) 229–312 (1995).
2. López-Arredondo, D. L., Leyva-González, M. A., González-Morales, S. I., López-Bucio, J. & Herrera-Estrella, L. Phosphate nutrition: improving low-phosphate tolerance in crops. Annu. Rev. Plant Biol. 65, 95–123 (2014).
3. Hollford, I. C. R. Soil phosphorus: its measurement and its uptake by plants. J. Exp. Bot. 57, 2049–59 (2006).
4. Cai, H., Xie, W., Zhu, T. & Lian, X. Transcriptome response to phosphorus starvation in rice. Mol. Gen. Genet. 279, 1469–1487 (2015).
5. Miura, K. & Hasegawa, P. M. Sumoylation and other ubiquitin-like post-translational modifications in plants. Annu. Rev. Plant Biol. 57, 535–539 (2012).
6. Schroeder, J. I. et al. Using membrane transporters to improve crops for sustainable food production. Nature. 497, 60–66 (2013).
7. Schroeder, J. I. et al. Transcriptomic analysis of metabolic changes by phosphorus stress in rice plant roots. Plant Cell Environ. 26, 1515–1523 (2003).
8. Wasaki, J. et al. Transcriptomic analysis indicates putative metabolic changes caused by manipulation of phosphorus availability in rice leaves. J. Exp. Bot. 57, 2049–59 (2006).
9. Cai, H., Xie, W., Zhu, T. & Lian, X. Transcriptome response to phosphorus starvation in rice. Acta Physiol Plant. 34, 327–341 (2012).
10. Cai, H., Xie, W. & Lian, X. Comparative analysis of differentially expressed genes in rice under nitrogen and phosphorus starvation stress conditions. Plant Mol. Biol. Rep. 31, 160–173 (2013).
11. Osny, X. et al. Diversity in the complexity of phosphate starvation transcriptomes among rice cultivars based on RNA-Seq profiles. Plant Mol. Biol. 83, 523–537 (2013).
12. Secco, D. et al. Spatio-Temporal Transcript Profiling of Rice Roots and Shoots in Response to Phosphate Starvation and Recovery. Plant Cell. 25, 4285–4304 (2013).
13. Rojas-Triana, M. et al. Role of posttranslational in the control of phosphate starvation responses in plants. J Integr Plant Biol. 55, 40–53 (2013).
14. Friso, G. & van Wijk, K. J. Posttranslational modifications in plant metabolism. Plant Physiol. 169, 1469–1487 (2015).
15. Miura, K. & Hasegawa, P. M. Sumoylation and other ubiquitin-like post-translational modifications in plants. Trends Cell Biol. 20, 223–232 (2010).
16. Miura, K. et al. The Arabidopsis SUMO E3 ligase SIZ1 controls phosphate deficiency responses. Proc. Natl. Acad. Sci. USA 102, 7760–7765 (2005).
17. Miura, K. et al. SIZ1 regulation of phosphate starvation-induced root architecture remodeling involves the control of auxin accumulation. Plant Physiol. 155, 1000–1012 (2011).
18. Park, H. C. et al. Functional characterization of the SIZ/PIAS-type SUMO E3 ligases, OsSIZ1 and OsSIZ2 in rice. Plant Cell Environ. 33, 1923–1934 (2010).
19. Li, Z. et al. Heterologous expression of OsSIZ1, a rice SUMO E3 ligase, enhances broad abiotic stress tolerance in transgenic creeping bentgrass. Plant Biotechnol J. 11, 432–445 (2013).
20. Wang, H. et al. OsSIZ1, a SUMO E3 ligase gene, is involved in the regulation of the responses to phosphate and nitrogen in rice. Plant Cell Physiol. 56, 2381–2393 (2015).
21. Thangasamy, S. et al. Rice SIZ1, a SUMO E3 ligase, controls spikelet fertility through regulation of anther dehiscence. New Phytol. 189, 869–882 (2011).
22. Wang, H. et al. OsSIZ1 regulates the vegetative growth and reproductive development in rice. Plant Mol. Biol. Rep. 29, 411–417 (2011).
23. Wang, X., Tang, H., Bowers, J. E., Feltus, F. A. & Paterson, A. H. Extensive concerted evolution of rice paralogs and the road to regaining independence. Genetics. 177, 1753–1763 (2007).
26. Zhou, J. et al. OsPHR2 is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. *Plant Physiol.* **146**, 1673–1686 (2008).
27. Guo, M. et al. Integrative Comparison of the Role of the PHOSPHATE RESPONSE1 Subfamily in Phosphate Signaling and Homeostasis in Rice. *Plant Physiol.* **168**, 1762–76 (2015).
28. Ruan, W., Guo, M., Wu, P. & Yi, K. Phosphate starvation induced OsPHR4 mediates Pi-signaling and homeostasis in rice. *Plant Mol. Biol.* **93**, 327–340 (2017).
29. Han, B., Xue, Y., Li, J., Deng, X. W. & Zhang, Q. Rice functional genomics research in China. *Philos Trans R Soc Lond B Biol Sci.* **362**, 1009–1021 (2007).
30. Wang, N. et al. Mutant resources for the functional analysis of the rice genome. *Mol Plant*. **6**, 596–604 (2013).
31. Zhang, Y. et al. Involvement of OsPHR1 in phosphate acquisition and mobilization facilitates embryo development in rice. *Plant J.* **82**, 556–569 (2015).
32. Wu, P., Shou, H., Xu, G. & Jian, X. Improvement of phosphorus efficiency in rice on the basis of understanding phosphate signaling and homeostasis. *Curr. Opin. Plant Biol.* **16**, 205–212 (2013).
33. Friso, G. & van Wijk, K. J. Posttranslational Protein Modifications in Plant Metabolism. *Plant Physiol.* **169**, 1469–1487 (2015).
34. Johnson, E. S. Protein modification by SUMO. *Annu. Rev. Biochem.* **73**, 355–382 (2004).
35. Catalá, R. et al. The Arabidopsis E3 SUMO ligase SIZ1 regulates plant growth and drought responses. *Plant Cell.* **19**, 2952–66 (2007).
36. Hay, R. T. SUMO: a history of modification. *Mol Cell.* **18**, 1–12 (2005).
37. An, G., Jeong, D. H., Jung, K. H. & Lee, S. Reverse genetic approaches for functional genomics of rice. *Plant Mol. Biol.* **59**, 111–123 (2005).
38. Kawahara, Y. et al. Improvement of the Oryza sativa Nipponbare reference genome using next generation sequence and optical map data. *Rice*. **6**, 4 (2013).
39. Jackson, S. A. Rice: The First Crop Genome. *Rice*. **9**, 14 (2016).
40. O’Malley, R. C. & Ecker, J. R. Linking genotype to phenotype using the Arabidopsis unimutant collection. *Plant J.* **61**, 928–940 (2010).
41. Wang, Y. H. How effective is T-DNA insertional mutagenesis in Arabidopsis? *Biochem Tech.* **1**, 11–20 (2008).
42. Overvoorde, P., Fukaki, H. & Beeckman, T. Auxin Control of Root Development. *Cold Spring Harb Perspect Biol.* **2**, a001537 (2010).
43. Saini, S., Sharma, J., Kaur, N. & Pati, P. K. Auxin: a master regulator in plant root development. *Plant Cell Rep.* **32**, 741–757 (2013).
44. Mai, C. D. Genes controlling root development in rice. *Plant Sci.* **7**, 30 (2014).
45. Da, S. F. E., Lopitz-Otsoa, F., Lang, V., Rodríguez, M. S. & Matthiesen, R. Strategies to Identify Recognition Signals and Targets of SUMOylation. *Biochem Res. Biol.* **2012**, 875148 (2012).
46. Park, H. J. et al. SUMO3 and SUMOylation in plants. *Mol Cells.* **32**, 305–316 (2011).
47. López-Torrejón, G., Guerra, D., Catalá, R., Salinas, J. & del Pozo, J. C. Identification of SUMO targets by a novel proteomic approach in plant(F). *J Integr Plant Biol.* **55**, 96–107 (2013).
48. Xu, P. & Yang, C. Emerging role of SUMOylation in plant development. *Plant Signal Behav.* **8**, e24727 (2013).
49. Chen, C. C. et al. Arabidopsis SUMO E3 ligase SIZ1 is involved in excess copper tolerance. *Plant Physiol.* **156**, 2225–2234 (2011).
50. Park, B. S., Song, J. T. & Seo, H. S. Arabidopsis nitrate reductase activity is stimulated by the E3 SUMO ligase AtSIZ1. *Plant Cell Physiol.* **50**, 175–185 (2009).
51. Lopez-Torrejón, G., Guerra, D., Catalá, R., Salinas, J. & del Pozo, J. C. Identification of SUMO targets by a novel proteomic approach in plants(F). *J Integr Plant Biol.* **55**, 96–107 (2013).
52. Wang, N. et al. Regulation of OsSPX1 and OsSPX3 on expression of OsSPX domain genes and Pi-starvation signaling in rice. *Plant Mol Biol*. **6**, 201–210 (2000).
53. Shin, B. S., Park, S., Kwon, Y. & Lee, S. Arabidopsis nitrate reductase activity is stimulated by the E3 SUMO ligase AtSIZ1. *Nat Commun.* **2**, 400 (2011).
54. Sun, S. et al. A constitutive expressed phosphate transporter, OsPhr11, modulates phosphate uptake and translocation in phosphate-replete plant. *Plant Physiol.* **159**, 1571–1581 (2012).
55. Wang, Z. et al. Regulation of OsSPX1 and OsSPX3 on expression of OsSPX domain genes and Pi-starvation signaling in rice. *J Integr Plant Biol.* **51**, 663–674 (2009).
56. Jung, K. H., Čao, P., Sharma, R., Jain, R. & Ronald, P. C. Phylogenomic databases for facilitating functional genomics in rice. *Rice*. **8**, 26 (2015).
57. Chandran, A. K. N. et al. Updated Rice Kinase Database RKD 2.0: enabling transcriptome and functional analysis of rice kinase genes. *Rice*. **9**, 40 (2016).
58. Cai, H. V. & Karlsson, D. T. Response and transcriptional regulation of rice SUMOylation system during development and stress conditions. *RMB rep.* **43**, 103–109 (2010).
59. Wang, Z. et al. A practical vector for efficient knockdown of gene expression in rice (Oryza sativa L.). *Plant Mol. Biol. Rep.* **22**, 409–417 (2004).
60. Upadhyaya, N. M. et al. Agrobacterium-mediated transformation of Australian rice cultivars Jarrah and Amaroo using modified promoters and selectable markers. *Aust. J. Plant Physiol.* **27**, 201–210 (2000).
61. Livak, K. J. & Schmittgen, T. D. Analysis of real-time gene expression data using real-time quantitative PCR and the 2−ΔΔCt method. *Methods.* **25**, 402–408 (2001).
62. Sambrook, J., Fritsch, E. & Maniatis, T. Molecular cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y. (1989).
63. Ames, B. N. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* **8**, 115–118 (1966).

Acknowledgements
This work was supported by Chinese National Natural Science Foundation (31672226), The National Key Research and Development Program of China (2016YFD0100700), National Program on R&D of Transgenic Plants (2016ZX08009-003-005), the Jiangsu Provincial Natural Science Foundation (BK20141367), Innovative Research Team Development Plan of the Ministry of Education (IRT1256) and 111 Project (number 12009). We thank Prof. Y. Chengwei and J. Jieming at South China Normal University for insightful suggestions and technical support, respectively during the course of this study. We also thank S. Vishwanathan for going through the manuscript critically.

Author Contributions
W.P. participated in planning and conducting the experiments, data interpretation and helped in writing the manuscript. B.F., H.A., H.W., H.Z., R.S., X.L., Y.S. and Z.Z carried out different experiments. A.J. participated in planning, contributed to data interpretation and helped in writing the manuscript. G.X. participated in planning the study. S.S. conceived the study, participated in planning, analysis of the data, and helped in writing the manuscript. All authors read and approved the final manuscript.

Additional Information
Supplementary information accompanies this paper at doi:10.1038/s41598-017-10274-5

Competing Interests: The authors declare that they have no competing interests.
Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2017