LETTER

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The protein kinase Pstol1 from traditional rice confers tolerance of phosphorus deficiency

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As an essential macroelement for all living cells, phosphorus is indispensable in agricultural production systems. Natural phosphorus reserves are limited[1], and it is therefore important to develop phosphorus-efficient crops. A major quantitative trait locus for phosphorus-deficiency tolerance, *Pup1*, was identified in the traditional *aus*-type rice variety Kasalath about a decade ago[2,3]. However, its functional mechanism remained elusive[4,5] until the locus was sequenced, showing the presence of a *Pup1*-specific protein kinase gene[6], which we have named phosphorus-starvation tolerance 1 (*PSTOL1*). This gene is absent from the rice reference genome and other phosphorus-starvation-intolerant modern varieties[7,8]. Here we show that overexpression of *PSTOL1* in such varieties significantly enhances grain yield in phosphorus-deficient soil. Further analyses show that *PSTOL1* acts as an enhancer of early root growth, thereby enabling plants to acquire more phosphorus and other nutrients. The absence of *PSTOL1* and other genes—for example, the submergence-tolerance gene *SUB1A*—from modern rice varieties underlines the importance of conserving and exploring traditional germplasm. Introgression of this quantitative trait locus into locally adapted rice varieties in Asia and Africa is expected to considerably enhance productivity under low phosphorus conditions.

Phosphorus (P) is of unequivocal importance for the production of food crops, and the demand for P fertilizer is increasing worldwide. In Asia, where rice is the main and sometimes the only source of calories, 40% of the rice is produced in rain-fed systems, with little or no water control and frequent occurrence of floods, droughts and other calamities. In addition, 60% (29 Mha) of the rain-fed lowland rice is produced on poor and problem soils[9,10] (Fig. 1a) that are constrained by a multitude of abiotic stresses and are naturally low in phosphorus or P fixing. Rice yields are therefore low[11] and, not surprisingly, poverty in these regions is among the highest in the world (http://www.ruralpovertyportal.org/web/guest/region). A lack of resources or limited access to P fertilizer are some of the constraints for poor farmers. There is a high risk that the situation will be further aggravated given that phosphate rock, the source of P fertilizer, is a finite and non-renewable resource that is

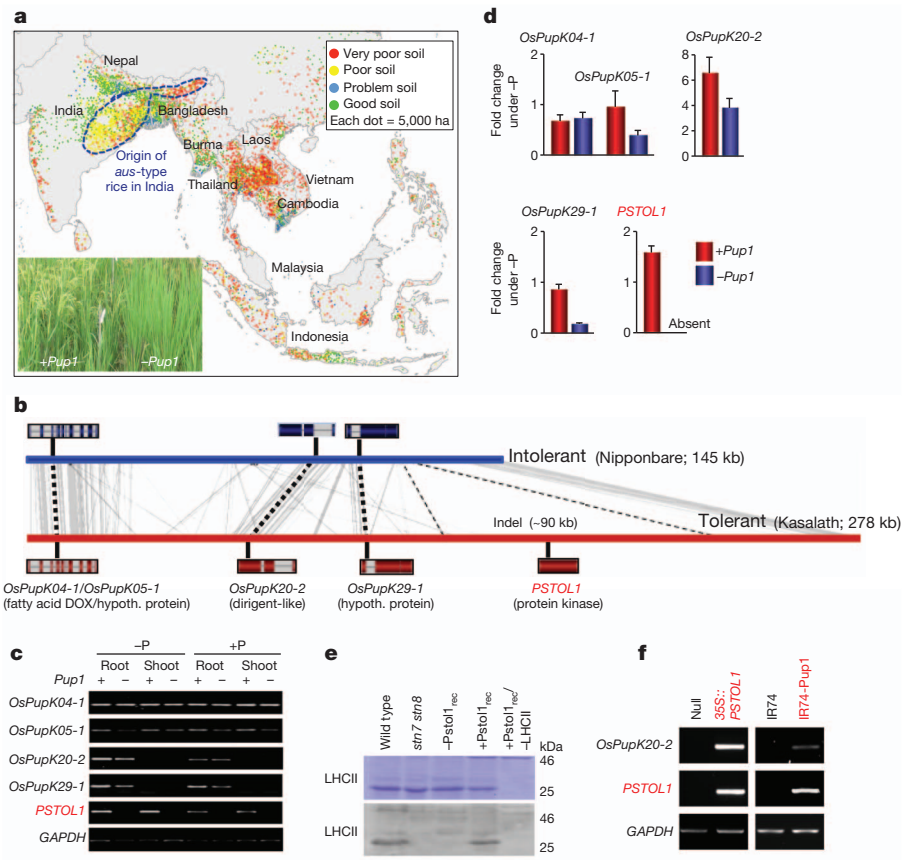

Figure 1 | Origin of the donor variety Kasalath and *Pup1* candidate genes. **a**, Problem soils in Asia and the origin of stress-tolerant *aus*-type rice varieties[9,10,13]. Inlay, breeding lines with and without the tolerant *Pup1* locus[8] under P-deficient field conditions. **b**, Relative position of *Pup1* candidate genes in Kasalath and the Nipponbare reference genome. *OsPupK05-1* is part of *OsPupK04-1* (refs 6, 8). DOX, dioxygenase. Hypoth., hypothetical. **c**, Semiquantitative RT–PCR analysis of *Pup1* candidate genes in contrasting Nipponbare NILs +*Pup1* and –*Pup1* grown in P-deficient soil +/– P fertilizer. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. **d**, qRT–PCR analysis of *Pup1* genes in roots of NILs (gene expression $+P = 1$). Error bars denote s.e.m. **e**, Top, gel stained with Coomassie blue. Bottom, phosphothreonine-specific immunoblot showing that recombinant Pstol1 protein restores phosphorylation of the light-harvesting complex II (LHCII) in the *Arabidopsis stn7 stn8* double mutant (lane 4). **f**, Semiquantitative RT–PCR analysis of *OsPupK20-2* in IR64 *35S::PSTOL1* plants and IR74-Pup1 NILs grown in +P hydroponics.

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concentrated in only a few countries (Morocco, China and the USA), and mining costs are rising[1,12]. Apart from the need for long-term strategies to address this problem, the development of rice varieties with high productivity under low P and other stress conditions is a valid and necessary approach to improve yield and enhance food security in rice-dependent countries.

In recent years, a specific group of rice (*aus*-type varieties) that originates from a region in India with poor and problem soils[9,10,13] (Fig. 1a) has been recognized as a valuable source of tolerance genes. For instance, the donor of the submergence-tolerance gene *SUB1A* is an *aus*-type variety and rice breeding lines with this gene (Sub1 or 'scuba' rice) survive up to 2 weeks in flooded fields[14,15]. Likewise, tolerance of drought and heat[16], in addition to other stresses, is present in such varieties. The *aus*-type variety Kasalath, which is tolerant of P deficiency, was identified about a decade ago, subsequently leading to the identification of a major quantitative trait locus (QTL) associated with P-deficiency tolerance[17]. At present, phosphorus uptake 1 (*Pup1*) is the only P-related QTL available for marker-assisted breeding programs, and tolerant Pup1 breeding lines have proven effective in field trials[7,8] (Fig. 1a). Previous efforts to link *Pup1* with known P-uptake-related mechanisms showed that *Pup1* near-isogenic lines (NILs) had improved root growth under stress, but the underlying mechanisms remained enigmatic[4], indicating that *Pup1* might act through a new mechanism or that the underlying gene may be missing in the reference genome.

Indeed, sequencing of the *Pup1* locus in Kasalath showed the presence of an ~90 kilobase transposon-rich insertion–deletion (indel) that is absent from the Nipponbare reference genome and other P-starvation-intolerant rice varieties[6] (Fig. 1b). A rice germplasm screen conducted with *Pup1*-specific molecular markers additionally showed that a gene located in the indel, the putative protein kinase gene *OsPupK46-2*, was most closely associated with tolerance of P deficiency and was highly conserved in stress-adapted rice accessions[7,8].

To gain insight into the function of *Pup1* and to identify the major genetic determinant of P-deficiency tolerance, the protein kinase *OsPupK46-2* and four additional *Pup1* candidate genes[8] were short-listed from the initially predicted 68 *Pup1* gene models[6]. Gene expression was analysed by semiquantitative reverse transcriptase PCR (RT–PCR) and quantitative (q)RT–PCR analyses in contrasting NILs with (+*Pup1*) and without (−*Pup1*) the Kasalath *Pup1* locus. The data confirmed that *OsPupK46-2* was absent from the Nipponbare genome and showed that it was upregulated under P-deficient conditions (Fig. 1c, d). Expression of the other genes did not change under −P conditions (*OsPupK04-1*, *OsPupK05-1*, *OsPupK29-1*) or increased in both +*Pup1* and −*Pup1* NILs (*OsPupK20-2*) (Fig. 1c, d). Additional analyses subsequently showed that *OsPupK20-2*, which codes for a dirigent protein, is downstream of the protein kinase (see below). On the basis of these data and the probable role of protein kinases in the sensing and signalling of P homeostasis[18,19], we considered *OsPupK46-2* the most obvious candidate gene and named it phosphorus-starvation tolerance 1 (*PSTOL1*).

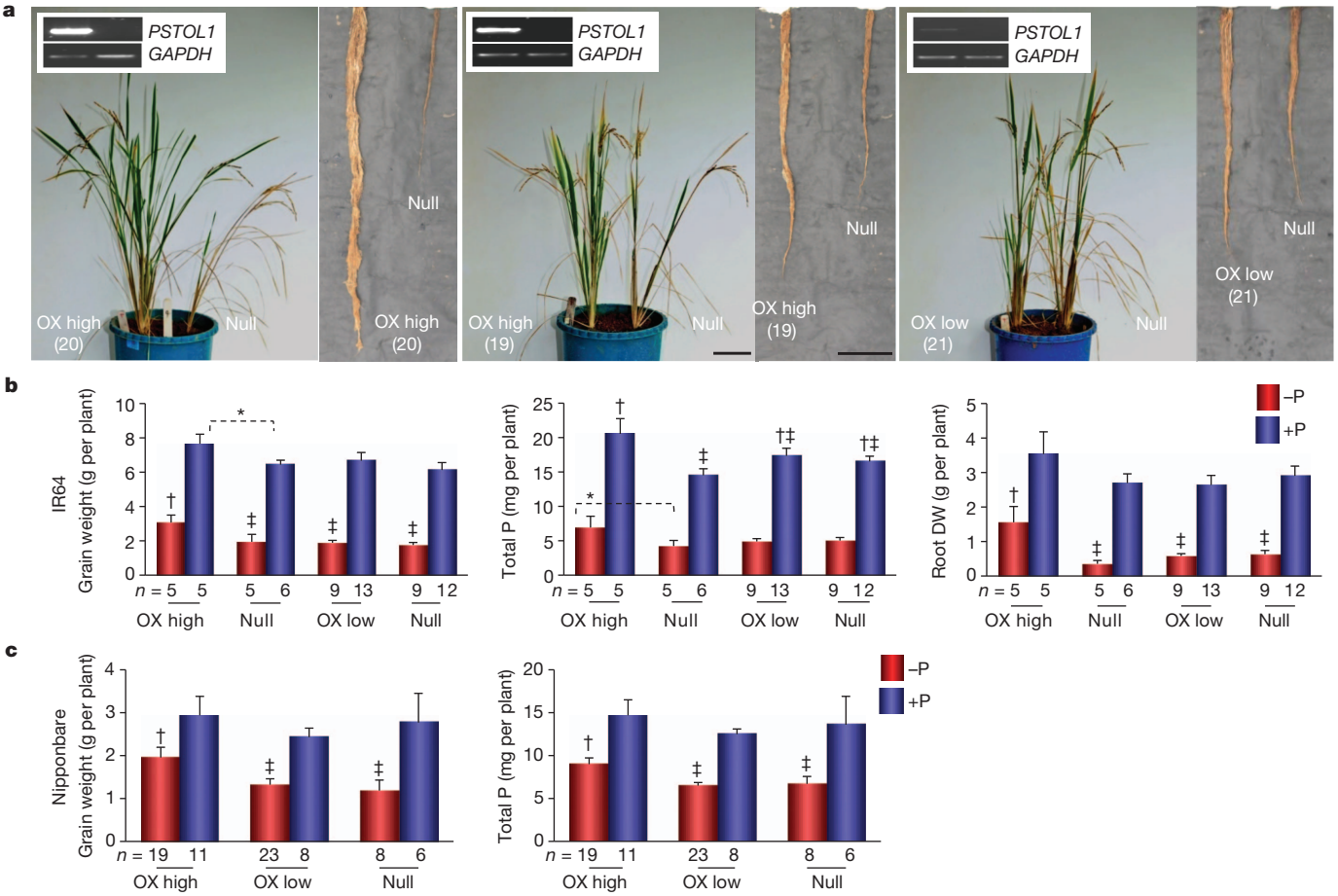

Figure 2 | *PSTOL1* overexpression enhances tolerance of P deficiency. **a**, Representative IR64 *35S::PSTOL1* plants with high (OX high) and low (OX low) transgene expression of independent events (event numbers 20, 19 and 21) and corresponding null segregants at 8 weeks in P-deficient soil (root photos were taken after harvest). **b**, Grain weight, P content and root dry weight (DW) of IR64 transformants and nulls. *n* = number of plants. **c**, Grain weight and total P content of Nipponbare transformants and nulls. Error bars indicate s.e.m. Significance ($P < 0.05$) is indicated by † (analysis of variance and Tukey's honestly significant difference test) and asterisks (paired *t*-test). The mean with symbol † indicates significant difference from the mean with symbol ‡. The means with the same symbol (†‡ versus †‡) or with a common symbol († versus †‡; ‡ versus †‡) are not significantly different from each other. Scale bars, 10 cm.

Pstol1 shows the highest amino acid sequence similarity with serine/threonine receptor-like kinases of the LRK10L-2 subfamily, but lacks the amino-terminal extension typically present in this family[20]. This classifies Pstol1 as a receptor-like cytoplasmic kinase[21]. Interestingly, the conserved kinase domain is most similar to the *Arabidopsis* defence-related receptor-like kinases PR5K[22] (58%) and SNC4 (ref. 23; 57%) (Supplementary Figs 1–3). To assess whether Pstol1 is a functional protein kinase, an *in vitro* phosphorylation assay was performed using thylakoid membranes isolated from the *Arabidopsis thaliana* double mutant *stn7 stn8*, which is defective in STN7 and STN8 (also known as AT1G68830 and AT5G01920, respectively) serine/threonine protein kinases and therefore devoid of phosphorylation of the light-harvesting complex II[24]. The data show that recombinant Pstol1 protein restored phosphorylation of *stn7 stn8* thylakoids to almost wild-type levels (Fig. 1e), confirming that Pstol1 is a functional serine/threonine protein kinase.

To quantify the effect of Pstol1 on plant performance under low-P stress, we generated transgenic plants with constitutive overexpression of the full-length *PSTOL1* coding region (*35S::PSTOL1*). Two rice varieties (IR64 and Nipponbare) were used for this experiment, representing two distinct types of modern irrigated varieties (*indica* and *japonica*, respectively) that naturally lack the *PSTOL1* gene[8] (Supplementary Fig. 4). Phenotypic analyses conducted in two different locations and P-deficient soil types (from fields that had not received P fertilizer for up to 40 years; −P) showed that high expression of the *PSTOL1* transgene enhanced grain yield by more than 60% under –P conditions in both varieties (Fig. 2a–c and Supplementary Fig. 5). Transgenic lines with low transgene expression were comparable to segregants without the transgene (null) that were used as controls. These data indicate that expression of *PSTOL1* above a certain threshold is required to confer tolerance of P deficiency. In both varieties, a significantly higher P content was observed in high *PSTOL1*-overexpressing lines (Fig. 2b, c). For the IR64 plants, we further confirmed that the superior performance of *PSTOL1* lines with high transgene expression was due to a higher root dry weight (Fig. 2a, b). The larger root system also enhanced the uptake of other nutrients, as nitrogen and potassium content were also higher in these lines (Supplementary Fig. 6). Subsequent phenotypic analyses of IR64 *PSTOL1*-overexpressing lines conducted in nutrient solution with high (100 μM) and reduced (10 μM) P concentrations showed that, under both P treatments, total root length and root surface area were significantly higher in transgenic seedlings (Fig. 3a, c). The same experiment was then repeated with two different contrasting *Pup1* NILs (IR64 and IR74, with (+) or without (–) *Pup1*) that were developed by marker-assisted introgression of the Kasalath *Pup1* locus[8]. In agreement with the above data, seedlings of +*Pup1* NILs

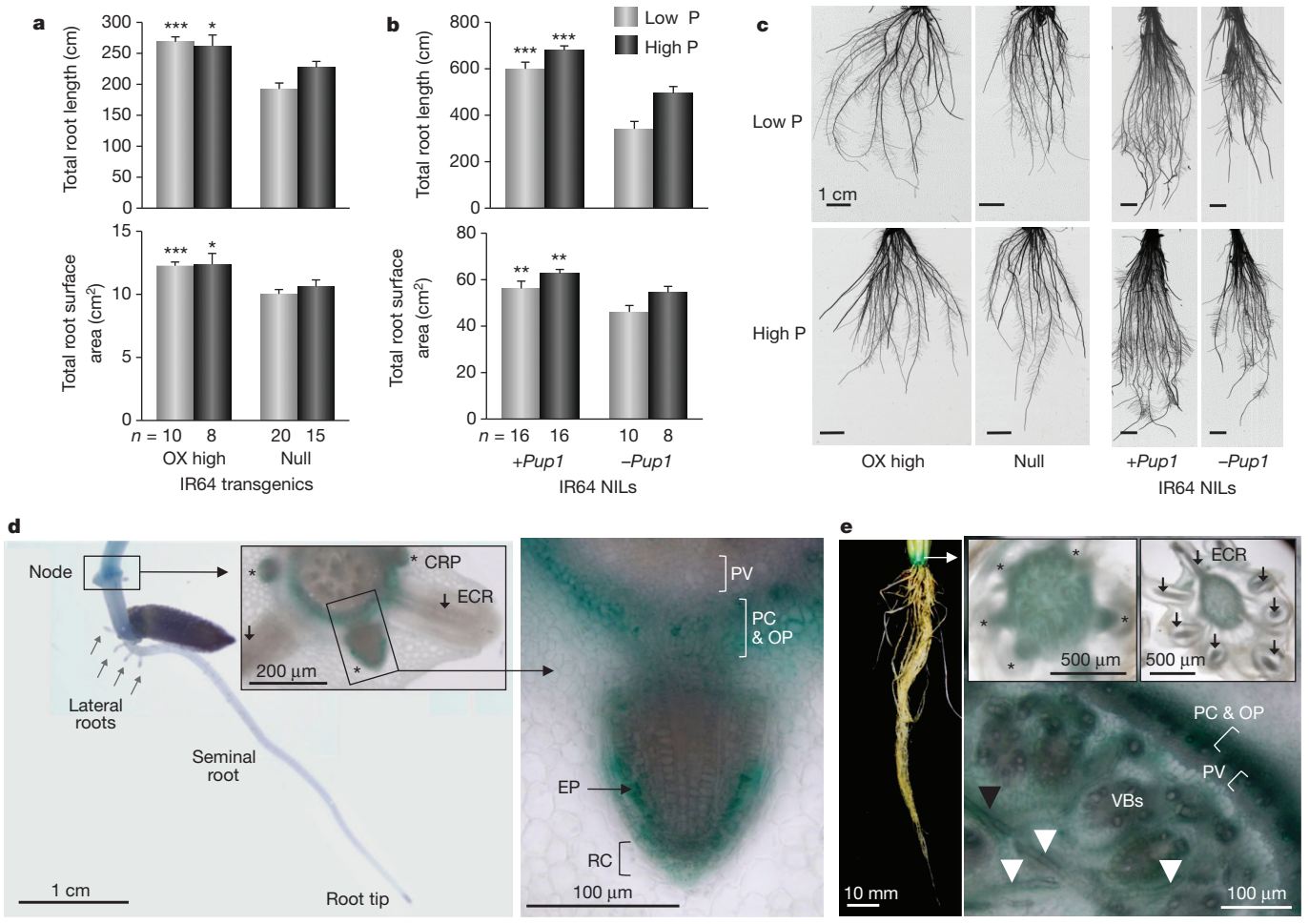

Figure 3 | *PSTOL1* is an enhancer of root growth. **a**, Total root length and surface area of IR64 *35S::PSTOL1* plants (OX high; T2 no. 20) and corresponding nulls grown in high-P (100 μM) and low-P (10 μM) hydroponics solution for 15 days. **b**, Root data of sister NILs with and without *Pup1* grown under the same conditions for 21 days. Error bars indicate standard error. Significance was analysed by paired *t* test (95%). *$0.05 > P \geq 0.01$; **$0.01 > P \geq 0.001$; ***$P > 0.001$). **c**, Representative root scans. Scale bars, 1 cm. **d**, GUS expression driven by the native *PSTOL1* promoter in young IR64 seedlings is observed in parenchyma (PC) and outer parenchyma (OP) cells adjacent to the peripheral vascular (PV) cylinder of the coleoptilar node and in crown root primordia (CRP; indicated by asterisks), but not in emerging crown roots (ECR; arrows). RC, root cap. **e**, GUS staining in older plants (28 days after germination) is likewise seen in crown root primordia (asterisks) and additionally in cells surrounding vascular bundles (VBs), which are interconnected by nodal vascular anastomoses (arrowheads).

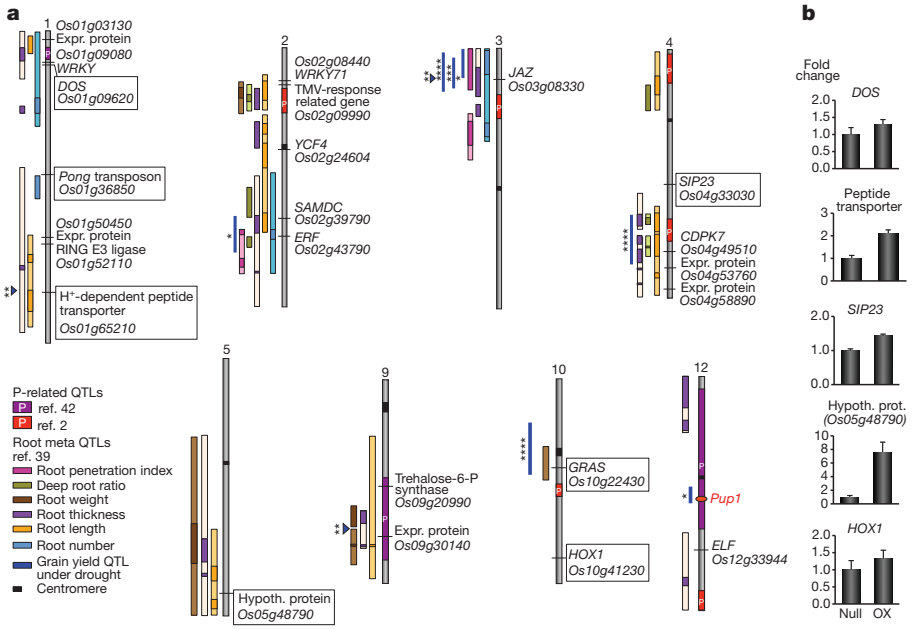

Figure 4 | *PSTOL1* putative downstream genes co-localize with root and drought QTLs. **a**, Approximate chromosomal location of genes with constitutively higher (boxed) or lower (all other genes) expression in roots of *35S::PSTOL1* transgenics. QTLs for P-deficiency tolerance are indicated in red and green on the chromosomes. Root-related meta-QTLs and QTLs for grain yield under drought are shown as colour-coded bars (see key). Centromeres are indicated in black. Expr., expressed. **b**, qRT–PCR analysis of upregulated genes in root samples of IR64 transgenics (OX) and null controls grown in +P hydroponics. References for the drought QTLs are *ref. 37, **ref. 38, ***ref. 40 and ****ref. 41.

showed significantly enhanced root growth under high and low P conditions (Fig. 3b, c and Supplementary Fig. 7). The finding that root growth was enhanced in *PSTOL1*-overexpressing lines as well as in *Pup1* introgression lines provides strong evidence that *PSTOL1* is indeed the major tolerance gene within the *Pup1* QTL and that this gene acts at least partially independently of P. In support of this, down-regulation of *PSTOL1* by RNA interference in Kasalath caused a significant reduction in root number and root surface area, which negatively affected overall plant growth (Supplementary Fig. 8a–g).

To analyse the expression of *PSTOL1* during root development in more detail, we expressed the β-glucuronidase (GUS) reporter gene under the control of the native *PSTOL1* promoter in transgenic IR64 plants. Specific GUS staining was detected in stem nodes where, in rice, crown roots are formed that constitute the main root system (Fig. 3d, e). Within the nodes, GUS staining was restricted to crown root primordia and parenchymatic cells located outside of the peripheral vascular cylinder. This expression pattern is similar to that described for other root-development genes, namely *CROWN ROOTLESS 1* (also known as *AB200234*) and *RR2*, a cytokinin type-A-responsive regulator[25,26]. In older plants, GUS staining was additionally detected in the cells surrounding the nodal vascular anastomoses, which interconnect vascular bundles (Fig. 3e). No GUS staining was observed in older, emerging crown roots or in the initial (seminal) seedling root. Taken together, our data indicate that *PSTOL1* is a regulator of early crown root development and root growth in rice.

Because Pstol1 is a protein kinase, it cannot directly regulate the expression of genes. However, regulation of transcription factors through phosphorylation has been well studied in the yeast P-starvation response system, which involves the transcription factor *PHO4* (ref. 18), as well as in two-component signalling systems that depend on membrane-bound histidine kinases[27]. To gain insight into the downstream responses of Pstol1, we conducted an Affymetrix gene-array analysis using root samples from soil-grown IR64 transgenic plants (high *PSTOL1* overexpression) and control plants. The data showed that known P-starvation genes were not differentially regulated in the transgenic plants (Supplementary Table 1). Similar results were obtained in a previous Agilent microarray analysis using *Pup1* NILs[5]. Instead, we identified 23 genes with constitutively (that is, independent of the P supply and developmental stage) higher or lower expression in the transgenic plants that are related to root growth and stress response (Supplementary Table 2). Interestingly, 21 of these differentially expressed genes co-localize with QTLs related to drought tolerance and root growth (Fig. 4a), providing further support for an important role of *Pup1*/*PSTOL1* during root development and stress tolerance. These findings are also supported by a *Pup1*-marker analysis that had shown high conservation of *PSTOL1* in drought-tolerant rice accessions[7,8]. In this context, we also determined that the *Pup1* dirigent gene (*OsPupK20-2*) is downstream of *PSTOL1*, as this gene was specifically induced in *35S::PSTOL1* plants and in +*Pup1* NILs (Fig. 1f).

To assess whether the expression of the genes identified is indeed independent of P and/or soil-related factors, a qRT–PCR analysis of selected genes was conducted using root RNA samples of *35S::PSTOL1* plants grown under high P conditions in hydroponics. Whereas the data were inconsistent for many of the downregulated genes (data not shown), higher expression was confirmed for six out of the seven genes that were specifically induced in *35S::PSTOL1* roots (Fig. 4b). Among these are two genes coding for transcription factors; namely *HOX1*, a positive regulator of root cell differentiation[28], and *DOS*, which was shown to delay leaf senescence in rice[29]. Altered expression of *HOX1* is well in agreement with a role for *PSTOL1* in root development. An association study further showed that a region on chromosome 1, where *DOS* and a gene coding for a WRKY-type transcription factor are located, was significantly associated with the presence of *PSTOL1* in a wider range of tolerant rice accessions (Supplementary Fig. 9). Interestingly, a putative peptide transporter was among the constitutively upregulated genes that might, in addition to P, improve the nitrogen status of the plants[30].

In light of the need to increase rice production for a growing population despite potentially negative impacts of climate change and increasing scarcity of natural resources, it will be critically important to systematically explore traditional rice varieties in which high-value genes such as *PSTOL1* are preserved, and to enable breeders to efficiently use these genes in breeding programs.

METHODS SUMMARY

***Pup1* candidate gene-expression analysis.** Expression of *Pup1* candidate genes was analysed by semiquantitative RT–PCR as described[8] and by qRT–PCR of root samples derived from 49-day-old plants of a set of Nipponbare NILs with and without the Kasalath *Pup1* introgression. Plants were grown in P-deficient and P-sufficient soil in Japan. Primer sequences for this and other experiments are provided in Supplementary Table 3.

Protein kinase activity of Pstol1. Recombinant Pstol1 protein was synthesized in *Escherichia coli* (strain BL21; pBAD-DEST49 expression vector, Invitrogen). Purified protein was incubated with isolated thylakoid membranes from the *Arabidopsis* double mutant *stn7 stn8* defective in phosphorylating the light-harvesting complex II[24].

Transgenic *35S::PSTOL1* plants and Affymetrix analysis. The *PSTOL1* coding sequence was cloned into the binary vector pMDC32 with the 35S promoter and used for *Agrobacterium*-mediated transformation of immature IR64 and Nipponbare embryos. Transgene copy number and expression level were determined by Southern blot analysis and RT–PCR, respectively. Independent transgenic lines (T1 and T2) were phenotyped at the International Rice Research Institute (the Philippines) and Japan International Research Center for Agricultural Sciences (Japan) in P-deficient soil +/− P fertilizer application (equivalent of 60 kg P_2O_5 ha^{-1}). For the Affymetrix gene-array analysis (line 20) root samples from IR64 *35S::PSTOL1* plants and nulls grown in P-deficient soil +/− P fertilizer were used. Plants were at the reproductive/heading stage (−P treatment) and at mid-tillering (+P control treatment). Complementary RNA samples were analysed at ATLAS Biolabs GmbH (Germany) using GeneChip Operating System 1.4.

Promoter::GUS analysis. A 1,755 base pair genomic region upstream of the *PSTOL1* start ATG codon was amplified from the genomic DNA of +*Pup1* NILs using the primer pair oRG107/oRG109 (Supplementary Table 3) and cloned into the pMDC164 vector containing the gene coding for GUS. For GUS staining, IR64 transgenic T1 plants were germinated on Petri dishes in the dark at room temperature (26 °C) and monitored for GUS after 1 week and 4 weeks.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions R.G. cloned and transformed the *PSTOL1* gene into IR64 and Nipponbare. R.G., J.P.T. and M.W. performed the phenotyping of transgenic plants. J.H.C. conducted the root meta-QTL analysis and J.H.C. and C.D. developed the IR64-Pup1 and IR74-Pup1 NILs. P.P. carried out the Pstol1 kinase assay. S.C. conducted the expression analysis of putative *PSTOL1* downstream genes. E.M.T.M. provided advice about the experiments and I.S.-L. provided technical support and infrastructure for rice transformation. R.G., M.W. and S.H. designed the experiments and wrote the manuscript.

Author Information GenBank protein accession numbers for *OsPupK04-1*, *OsPupK05-1*, *OsPupK20-2*, *OsPupK29-1* and *PSTOL1/OsPupK46-2* are BAH79993, BAH79994, BAK26565, BAH80018 and BAK26566, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to S.H. (s.heuer@cgiar.org).

METHODS

qRT–PCR of *Pup1* candidate genes. Seeds of NILs segregating for the *Pup1* locus (+*Pup1*, NILs 6-4, Y-4 and 14-4; –*Pup1*, NILs Y6, Y10 and Nipponbare)[7,17] were sown directly in pots filled with P-deficient and P-fixing andosol from a field located at Tsukuba, Japan, that had not received P fertilizer throughout its 40-year cropping history (–P). An equivalent of 60 kg P ha^{-1} was applied for the control treatment (+P). Pots were initially watered every 2–3 days and afterwards the soil was kept near field capacity. The experiment was conducted in a completely randomized design with three replications and four plants per replicate pot. Root tissue samples were taken at 49 days after sowing. Total RNA was extracted using the RNeasy mini kit according to the instructions of the manufacturer (Qiagen) and treated with RNase-free DNase I (Qiagen). qRT–PCR was performed as described[5] with some modifications. Complementary DNA synthesis was conducted at 37 °C for 15 min followed by 5 s of 85 °C using 500 ng DNase-treated total RNA with PrimeScript RT reagent kit (Takara). qRT–PCR was performed with 10 ng reverse transcriptase template and SYBR Premix Ex Taq (Perfect Real Time; Takara). PCR cycle conditions were 94 °C for 10 s as the first denaturing step, followed by 40 cycles at 94 °C for 5 s, 55–60 °C for 10 s and 72 °C for 15 s, and a gradual increase in temperature from 55 °C to 96 °C during the dissociation stage to monitor the specificity of each primer pair. Rice *18S* (also known as *RRN18*) was used as an internal control. For primer sequences, see Supplementary Table 3. Expression levels were calculated using the delta-delta comparison and expressed as fold changes under –P relative to expression under +P conditions (expression = 1).

***In vitro* phosphorylation assay.** Seeds of *A. thaliana* ecotype Col-0 and of the *stn7 stn8* double mutant were sown in plastic trays containing one portion of Techinc and one portion of Flox 6 soils and incubated for 3 days at 5 °C in the dark to break the dormancy. Plants were grown in a greenhouse under long-day conditions (16 h light and 8 h dark) for 4 weeks. Thylakoids were isolated from 4-week-old plants as described[24] in the presence of the phosphatase inhibitor sodium fluoride (10 mM). The coding sequence (CDS) of *PSTOL1* was cloned into the pBAD-DEST49 vector (Invitrogen), and recombinant Pstol1 ($Pstol1_{rec}$) was expressed in the *E. coli* strain BL21 with a carboxy-terminal 6× His-tag. $Pstol1_{rec}$ was purified under denaturing conditions following a nickel-nitrilotriacetic acid batch purification procedure according to the instructions of the manufacturer (Qiagen). After protein precipitation in 10% trichloroacetic acid followed by three washing steps with absolute ethanol, around 500 µg of $Pstol1_{rec}$ protein was re-suspended in 500 µl 1% (w/v) lithium dodecyl sulphate, 12.5% (w/v) sucrose, 5 mM e-aminocaproic acid, 1 mM benzamidine and 50 mM HEPES potassium hydroxide buffer, pH 7.8, as previously described[31]. Subsequently, $Pstol1_{rec}$ protein was boiled for 2 min at 100 °C and incubated for 15 min at 25 °C. Dithiothreitol (DTT; 75 mM final concentration) was then added and the solution was subjected to three freeze–thaw cycles (20 min at –20 °C, 20 min at –80 °C and 20 min at −20 °C, thawing in an ice-water bath, and 5 min at 25 °C). After completion of the three freeze–thawing cycles, octylglucopyranoside (1% (w/v) final concentration) was added and the solution was kept on ice for 15 min before potassium chloride (75 mM, final concentration) was added to precipitate the lithium dodecyl sulphate detergent. After centrifugation at 16,000g at 4 °C for 10 min, the supernatant containing the re-folded $Pstol1_{rec}$ in the presence of 1% (w/v) octylglucopyranoside was collected. Subsequently, 1 µl of kinase was incubated together with thylakoids corresponding to 5 µg of total chlorophyll. The phosphorylation reaction was performed in 50 µl total volume containing 0.06% (w/v) dodecyl-B-D-maltoside, 5 mM magnesium acetate, 5 mM DTT, 100 mM HEPES potassium hydroxide, pH 7.8, 200 mM ATP and 10 mM sodium fluoride at 37 °C for 2 h. The reaction mixture was loaded on an SDS–PAGE, and immunoblot analyses with phosphothreonine-specific antibodies (Cell Signaling) were performed as described[32]. A replicative SDS–PAGE was stained with Coomassie blue.

Generation of *35S::PSTOL1* transgenic plants. The CDS of *PSTOL1* was amplified from Kasalath genomic DNA using the primer pair oKas4603 and oKas4604 (all primer sequences are provided in Supplementary Table 3), cloned into pCR8/GW/TOPO TA cloning vector (Invitrogen) and sent for sequencing (Macrogen). Through LR clonase recombination reaction (Invitrogen), the CDS was sub-cloned into the pMDC32 binary destination vector[33] containing the 35S promoter and NOS terminator (*35S::PSTOL1*). The construct was sequenced using primer pairs amplifying the 35S promoter (oRG89) and the NOS-terminator (oSH07) with adjacent CDS, respectively. The correct sequence of *PSTOL1* was re-confirmed by sequencing with the primer pair oKas4603 and oKas4604. Transformation of the construct into the *indica*-type IR64 and *japonica*-type Nipponbare rice varieties, which naturally lack the *PSTOL1* gene, was mediated by the *Agrobacterium tumefaciens* strain LBA4404 according to a published protocol[34] with modifications (I.S.-L. *et al.*, in preparation). Transgenic plants were tested by genomic PCR in the T1 generation for the presence of the hygromycin phosphotransferase gene (*hpt*; primer pair oRG127 and oRG128) and the 35S promoter with part of the CDS (primer pair oRG89 and oRG88). PCR was carried out in a total volume of 20 µl with the following conditions: 100 ng genomic DNA, primers (0.2 µM each of forward and reverse), 1× PCR buffer, 0.5 mM dNTP mix and 1.5 U iTaq DNA polymerase (Intron Biotechnology). The PCR cycle settings were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, Ta (55 °C for primer pair oRG127 and oRG128, 60 °C for primer pairs oRG88 and oRG89 and GAPDH forward (-F) and GAPDH reverse (-R) for 30 s, 72 °C for extension time (30 s for oRG127 and oRG128 and 45 s for oRG88 and oRG89 and GAPDH-F and GAPDH-R) and a final extension at 72 °C for 10 min. As a control, the GAPDH gene was amplified using the primer pair GAPDH-F and GAPDH-R. PCR products were separated by agarose gel electrophoresis and stained with SYBR Safe (Invitrogen). The copy number of the transgene in selected plants was determined by Southern blot analysis using genomic DNA digested with XbaI and SacI, respectively, and hybridized with a digoxigenin-labelled *hpt* probe. Plants with independent transformation events were selected for phenotypic analysis in the T1 generation (Supplementary Fig. 4).

Phenotyping of *35S::PSTOL1* plants. T1 seeds from selected independent IR64 transgenic lines (Supplementary Fig. 4b) were pre-germinated in Petri dishes for 3 days in the dark at room temperature (26 °C) before seedlings were transferred into seedling trays. At 21 days after germination (DAG), transgenic plants and the corresponding null segregant were transferred into pots filled with P-deficient soil (P-Bray, 1.23 ± 0.30 mg kg^{-1}; P-Olsen, 0.77 ± 0.46 mg kg^{-1}) from Siniloan (Luzon, the Philippines). To control for pot-to-pot variation, one transgenic plant and one null segregant were always grown together in each pot. Each pot received the equivalent of 90 kg nitrogen ha^{-1}, 40 kg potassium ha^{-1} and 20 kg zinc ha^{-1}. The equivalent of 60 kg P ha^{-1} was applied only to the +P control treatment that was done in parallel. To mimic upland field conditions, plants grown under –P conditions were exposed to a dry-down treatment until leaf rolling at about 60 days after germination. Control pots were kept well watered but aerobic.

In an initial experiment, seven independent lines and the corresponding nulls were analysed (data not shown) and two lines (19 and 20) with high transgene expression and three lines (5, 14 and 21) with low transgene expression (Supplementary Figs 4 and 5) were selected for detailed analyses. A similar phenotyping experiment was conducted at the Japan International Research Center for Agricultural Sciences using independent T2 Nipponbare transgenic lines grown in well-watered (aerobic) P-deficient soil from Tsukuba (Japan) (Supplementary Fig. 4a). For the +P control, soil from a field that had regularly received P fertilizer was used and 60 kg P h^{-1} was additionally applied.

Macronutrients in roots, shoots and grains of IR64 transgenic plants and null controls were analysed by the Analytical Services Laboratory (ASL) at the International Rice Research Institute. The Kjeldahl method was used to determine the percentage of nitrogen, whereas a modified ASL nitric/perchloric acid digestion was done for inductively coupled plasma analysis of phosphorus and potassium.

Semiquantitative RT–PCR analysis of transgene expression. RT–PCR analysis of *35S::PSTOL1* expression was conducted using leaf samples. Total RNA was extracted using Trizol (Invitrogen) or RNeasy mini kit (Qiagen) and DNA contaminations were removed with RNase-free DNase I (Promega or Qiagen). cDNA synthesis in the IR64 experiment was performed at 55 °C for 1 h in a 20 µl reaction with 1 µg RNA template, 2.5 µM oligo dT, 0.5 mM dNTP mix, 0.01 M DTT, 1× first-strand buffer and 200 U of Superscript III RT (Invitrogen). For the Nipponbare experiment, 500 ng RNA template was used for cDNA synthesis in a total volume of 10 µl using PrimeScript RT reagent kit (Takara) at 37 °C for 15 min and then 85 °C for 5 s. For standard PCR analyses, 0.5–1 µl cDNA was used as template for amplification of the transgene with iTaq DNA polymerase (Intron Biotechnology) or Takara Taq (Takara) using gene-specific primers (0.2 µM each of oKas4603 and oKas4604; Supplementary Table 3). *GAPDH* was used as a positive control.

Root scan of IR64 *35S::PSTOL1* T2 plants and *Pup1* NILs grown in hydroponics. Seeds of the IR64 T2 transgenic line 20 and seeds of IR64-Pup1 and IR74-Pup1 NILs[8] were pre-germinated in Petri dishes in the dark at room temperature. After 3 days, germinated seeds were transferred to Yoshida culture solution[35] with 100 µM and 10 µM NaH_2PO_4, respectively. The solution was replaced every 3 days. Total root length and root surface area of seedlings (11–21 DAG) were measured using WinRhizo (MAC STD1600; Regent Instruments). Each root system was evenly spread out and scanned at least twice to obtain average values. Each experiment was reproduced at least once. Null controls and NILs without *Pup1* were always grown and analysed in parallel.

***PSTOL1* promoter::GUS IR64 transgenic plants.** The 1,755 base pair promoter of *PSTOL1* was amplified from the genomic DNA of +*Pup1* NILs using the primer pair oRG107 and oRG109 (Supplementary Table 3), cloned into the pCR8/GW/TOPO TA cloning vector (Invitrogen) and sent for sequencing (Macrogen). The promoter fragment was sub-cloned into a pMDC164 binary destination vector[31]

through LR clonase recombination reaction (Invitrogen). The final construct contained the *GUS* gene driven by the *PSTOL1* promoter, which was confirmed using the forward primer oRG120, sequencing from the 3′ end of the promoter extending to the CDS of the *GUS* gene. The construct was transformed into IR64 using the same protocol as described above. Transformed T0 plants were identified by genomic PCR using oRG120 and oRG134, also verifying the fusion of the *PSTOL1* promoter with the *GUS* gene. PCR conditions were the same as described above. For expression analyses, 1-week-old T1 seedlings grown in Petri dishes at room temperature in the dark were incubated in GUS staining solution according to the protocol[36]. Samples were stored in 70% ethanol before embedding in agarose for sectioning (200 μm) and brightfield microscopy (Olympus BX53 with attached Olympus DP70 camera).

Affymetrix gene-expression analysis. For microarray analyses, root samples of IR64 *35S::PSTOL1* and the corresponding null segregants were collected from T1 plants of line 20 grown in pots with P-deficient soil under stress (–P; dry-down) and control (+ P fertilizer; well-watered aerobic) conditions. Plants grown under control conditions were sampled at the four-tiller stage at 33 DAG. The stress treatment delayed development, and plants were collected at the heading stage when plants had developed two to four tillers. For all treatments, samples of two biological replicates were analysed. Total root RNA was extracted using Trizol according to the instructions from the manufacturer (Invitrogen), with modifications. The RNA was re-precipitated by adding 2.5× volume absolute ethanol and one-tenth volume 3M NaOAc, pH 5.2, washed twice (70% and 100% ethanol), air-dried and dissolved in RNase-free water before treatment with RNase-free DNase I (Promega). cRNA synthesis and labelling, hybridization and data analysis with the GeneChip operating system 1.4 were performed by ATLAS Biolabs GmbH using Affymetrix GeneChip rice genome arrays. Identification of genes with differential expression between transgenics and nulls and between P treatments was restricted to probe-set IDs with consistent data in both replicates. For the identification of genes with lower expression in transgenic plants compared with nulls, all IDs 'present' (expressed) in the nulls were used. For the identification of genes with higher expression, all IDs present in transgenic plants were used. Genes classified as 'constitutively' changed in the transgenics showed significantly ($P < 0.05$) altered expression in all data sets.

Expression analysis and physical location of putative *PSTOL1* downstream genes. For qRT–PCR analysis of the genes indentified in the Affymetrix study, roots from IR64 *35S::PSTOL1* T2 and null control plants grown hydroponically in Yoshida culture solution with 100 μM P were collected at 49 DAG. Total RNA extracted with Trizol (Invitrogen) was treated with RNase-free DNase I (Promega) and cDNA synthesis was performed with Transcriptor First Strand cDNA Synthesis Kit (Roche) using 1 μg DNase-treated RNA. qRT–PCR was conducted with LightCycler 480 SYBR Green I Master (Roche) using 0.5 μl cDNA template with the following PCR conditions: 94 °C for 5 min, 40 cycles at 94 °C for 10 s, 55 °C for 5 s and 72 °C for 20 s. Primer sequences are provided in Supplementary Table 3. *GAPDH* was used as an internal control. Expression levels were calculated using the delta-delta comparison and expressed as fold change relative to the expression in null controls (expression = 1). The physical location of genes was derived from the Rice Genome Browser (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/) and the physical position of drought tolerance and meta-QTLs for roots and drought was derived from published data[2,37–42]. The data were manually summarized and graphically illustrated.

Association analysis. A total of 79 rice varieties with different *Pup1* haplotypes[8] were genotyped with 379 single-nucleotide polymorphism (SNP) markers using the RiceOPA2.1 BeadXpress platform[43] and analysed using structure[44] to identify co-ancestry subgroups. The optimum number of populations (K) was selected by testing for $K = 1$ to $K = 8$ using ten independent runs of 10,000 burn-in runs followed by 100,000 iterations with a model allowing for admixture and correlated allele frequencies[45]. $K = 6$ provided the best distinction and two subgroups with the most contrasting *Pup1* haplotypes (Kasalath type, +*Pup1*; Nipponbare type, –*Pup1*) were selected for further analysis. SNP markers located within the putative *PSTOL1* downstream genes (Fig. 4) are not present in the 379 SNP set and markers located within approximately 1 megabase distance from the genes were therefore used for analysis of allelic associations with *PSTOL1* using TASSEL 3.0[46] (Supplementary Fig. 9). Rice accessions included in this study were: Kas group, Kasalath, AUS196, AUS257, Dular, IR84144-11-12, Lemont and Vandana; Nip group, Bala, CT6510-24-1-2, IR 42, IR64, IR66424-1-2-1-5, IR73678-6-9-B, IR 74, IR74371-46-1-1, K36-5-1-1BB, Nipponbare, PM-36 and Vary Lava 701.

RNA interference (RNAi) transgenic plants. A 322 bp fragment specific to the *PSTOL1* gene was amplified using the primer pair oSH07 and oSH08 and cloned into pENTR/D-TOPO vector (Invitrogen). The cloned fragment was transferred into pANDA RNAi vector[47] through LR clonase recombination reaction (Invitrogen). The RNAi construct was transformed into the *Pup1* donor variety Kasalath using the rice-transformation protocol described above. Six RNAi lines (T2 and T3 generation) were selected on the basis of semiquantitative RT–PCR showing downregulation of *PSTOL1* in roots using the oSH07 and oSH08 primer pair as described above. To verify whether the RNAi cassette is active, the expression of the *GUS* linker between the sense and antisense sequence[47] of the cloned *PSTOL1* fragment was determined. Selected RNAi lines were grown in hydroponics culture solution and in P-deficient soil and phenotyped (see supplementary Fig. 8). Wild-type Kasalath and null segregants were analysed in parallel.

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