Functional characterization of the PHT1 family transporters of foxtail millet with development of a novel Agrobacterium-mediated transformation procedure

S. Antony Ceasar1,2, Alison Baker1 & S. Ignacimuthu2

Phosphate is an essential nutrient for plant growth and is acquired from the environment and distributed within the plant in part through the action of phosphate transporters of the PHT1 family. Foxtail millet (Setaria italica) is an orphan crop essential to the food security of many small farmers in Asia and Africa and is a model system for other millets. A novel Agrobacterium-mediated transformation and direct plant regeneration procedure was developed from shoot apex explants and used to downregulate expression of 3 members of the PHT1 phosphate transporter family SiPHT1;2 SiPHT1;3 and SiPHT1;4. Transformants were recovered with close to 10% efficiency. The downregulation of individual transporters was confirmed by RT-PCR. Downregulation of individual transporters significantly reduced the total and inorganic P contents in shoot and root tissues and increased the number of lateral roots and root hairs showing they have non-redundant roles. Downregulation of SiPHT1;2 had the strongest effect on total and inorganic P in shoot and root tissues. Complementation experiments in S. cerevisiae provide evidence for the ability of SiPHT1;1, 2;1, 2;1,3, 1;7 and 1;8 to function as high affinity Pi transporters. This work will aid development of improved millet varieties for global food security.

Received: 21 July 2017
Accepted: 10 October 2017
Published online: 25 October 2017

1Centre for Plant Sciences and School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, LS2 9JT, UK. 2Division of Plant Biotechnology, Entomology Research Institute, Loyola College, Chennai, 600034, India. Correspondence and requests for materials should be addressed to S.A.C. (email: antony_sm2003@yahoo.co.in)
oocytes. PHT1 transporters are found to have 12 transmembrane segments and belong to the Major Facilitator Superfamily (MFS) of transporters.6–7

Millets are important cereals used as a food and feed in developing countries of Asia and Africa. Foxtail millet (Setaria italica) is one of the important millets mostly cultivated in the semi-arid regions of Asia and Africa and has been used as an excellent model species for various genetic studies.8,9 Together with its wild relative green foxtail (Setaria viridis), it is the only millet with its whole genome sequenced.8,10 While the genome sequencing of other millets like finger millet and pearl millet are still in progress due to their larger genome size,11 foxtail millet has been considered as an ideal millet to study the functions of PHT1 transporters. Although millets are important cereal crops in less developed countries, they have not been paid much attention for genetic transformation studies.12 Even only a few reports are available on the Agrobacterium-mediated transformation of foxtail millet13–15. However, in all these reports immature inflorescence was used as initial explants and callus mediated regeneration was adopted to recover the transgenic plants. The callus mediated regeneration is a time consuming process and may also induce some clonal variations. Hence, development of rapid regeneration protocol is needed for this important model crop following an efficient Agrobacterium-mediated transformation.

In a previous study, we have analysed the expression patterns of 12 PHT1 transporters of foxtail millet (SiPHT1;1 to SiPHT1;12) as a function of external Pi concentration and in response to colonisation of arbuscular mycorrhizal fungus (AMF) Funneliformis mosseae. The transporter SiPHT1;2 has been found to express in all tissues and at all stages of growth tested while SiPHT1;4 was found to be induced by low Pi stress in root tissues. Many transporters were also induced in 15 days old shoot tissues under low Pi condition. Transports SiPHT1;8 and SiPHT1;9 have been found to be induced by AMF colonisation in root tissues. However, functions of these transporters have not yet been studied.

In this article we report a novel Agrobacterium-mediated transformation system using shoot apex explants with a direct plant regeneration procedure. Yeast complementation studies of some of these transporters was performed in a Saccharomyces cerevisiae pho84 mutant and the in planta functions of 3 transporters (SiPHT1;2, 1;3 and 1;4) studied by downregulation through RNA interference (RNAi). This study will help to understand the role of PHT1 transporters on Pi transport in foxtail millet with a view to improve Pi uptake and efficient utilisation under low Pi stress conditions. The efficient Agrobacterium-mediated transformation system reported in this study may be useful for other millets and closely related wild relative like green foxtail (Setaria viridis).

Results

PHT1 transporters of foxtail millet can complement the low phosphate growth deficiency of a PHO84 deficient yeast mutant. To determine whether the SiPHT1 family encodes functional phosphate transporters, six members (SiPHT1;1, 1;2, 1;3 1;4 1;7 and 1;8) were tested for the ability to complement the growth of a S. cerevisiae Δpho84 mutant under high (1.0 mM) and low (0.2 mM) Pi. The cells were initially grown on 1 mM Pi with the addition of 2% galactose for the induction of expression of the transporter genes. On 1 mM Pi, all strains, including the empty vector transformed (Fig. 1A, GFP2 dotted line) showed similar growth curves to the positive control (Fig. 1A, PHO84 solid line) reaching stationary phase by 20 h with an OD600 between 1 and 1.5, showing that all strains were viable (Fig. 1A). When the experiment was repeated using 0.2 mM Pi, as expected, the empty vector control (Fig. 1B, GFP2 dotted line) barely grew. In contrast the mutant transformed with endogenous PHO84 gene (Fig. 1B, solid line) grew better than the negative control, reaching an OD600 of 0.18 after 20 h. With the exception of SiPHT1;4, the cells transformed with the SiPHT1 transporters grew similarly to, or in the case of the SiPHT1;2 transporter even better than, those transformed with the endogenous PHO84 transporter (Fig. 1B).

Cells transformed with empty vector (GFP2-negative control), native PHO84 (PHO84 positive control) and SiPHT1;2 were further examined by growing them in different concentrations of Pi (10, 50, 300 and 500 μM Pi) (Fig. 1C). Cells transformed with either of PHO84 or SiPHT1;2 grew much better than the negative control, even at 10 μM Pi, with the difference being more marked at 50 and 300 μM. At 500 μM, the negative control could grow but still to a much lesser extent than the positive control and SiPHT1;2, whereas in 1 mM Pi the positive and negative controls grew similarly (Fig. 1A). Thus all the transporters tested with the possible exception of SiPHT1;4 could complement the S. cerevisiae Δpho84 mutant for growth on 0.2 mM Pi.

Development of a novel direct plant regeneration and Agrobacterium-mediated transformation systems. Whilst heterologous complementation is a convenient test for functionality of phosphate transporters, to understand their roles within the physiology of the plant phosphate response requires manipulation of expression in planta. To this end an Agrobacterium-mediated transformation and novel direct regeneration protocol was developed for foxtail millet. Shoot apex explants were initially cultured on Murashige and Skoog (MS) medium containing various concentrations of cytokinins benzylamino purine (BAP), thidiazuron (TDZ) or kinetin (KN) at varied concentrations (Fig. 2A; Table 1). Multiple shoot induction was seen in all phytohormone containing media after 2 weeks of incubation, with significantly higher number of shoots per explant (28 ± 0.89) in the MS medium containing 0.5 mg/l BAP, after 4 weeks of incubation in the light (Table 1; Fig. 2B). Based on this study, MS medium containing 0.5 mg/l BAP was used in subsequent experiments as the shoot induction medium (SIM). The shoot clumps with multiple shoots obtained from MS medium containing 0.5 mg/l BAP were transferred onto the shoot elongation medium (SEM) containing MS salts alone devoid of phytohormones for shoot elongation. The shoot clumps cultured on the SEM elongated to produce the normal plants within 2 weeks (Fig. 2C). The shoots were very healthy and green in appearance. The plants were rooted in the same medium (SEM) after 3 weeks of incubation (Fig. 2D). After transfer to vermiculite and maintenance under polythene for a further 2 weeks for hardening (Fig. 2E and F), the plants were moved to the greenhouse with 100% survival rate and grown to maturity (Fig. 2G and H). The plants were grown up to seed setting and no variation in regenerated
plants for growth, flowering and seed setting was observed when compared to the wild plants (non-tissue culture) grown from seeds.

The direct plant regeneration system developed here was utilized for the subsequent Agrobacterium-mediated transformation of foxtail millet with RNAi vectors (pFGC-SiPHT1;2, pFGC-SiPHT1;3 or pFGC-SiPHT1;4) (Supplementary Figures S1–S3) and recovery of stable transgenic plants. The stepwise protocol for Agrobacterium-mediated transformation is outlined in Fig. 3. Three days old shoot apex explants were found to be good source for the transformation of foxtail millet based on this study. The explants were infected with Agrobacterium for 10–15 mins. This time period was found to be sufficient for the attachment of Agrobacterium cells onto the shoot apex explants. Explants were co cultivated on filter paper without directly touching the media (to reduce moisture) for 3 d in dark (Fig. 4A), then transferred onto the SIM containing 25 mg/l hygromycin and 250 mg/l cefotaxime. Screening experiments with a range of hygromycin concentrations showed the explants were effectively killed at 20 mg/l or above (Supplementary Figure S4) so 25 mg/l was chosen for selection of

---

Figure 1. Complementation of S. cerevisiae pho84 mutant with foxtail millet PHT1 transporters. Growth curves of S. cerevisiae pho84Δ mutant transformed with the indicated foxtail millet SiPHT1 transporters, empty vector (GFP-2 dotted line) and S. cerevisiae PHO84 (solid line) under 1.0 mM Pi (A) and 0.2 mM Pi (B). Assay of growth of pho84Δ mutant cells transformed with SiPHT1;2, ScPHO84 or empty vector (GFP-2) in different concentrations of Pi (C). The OD_{600} was measured after 20 hrs of growth. All the values are expressed as mean ± SD of 3 replicates and 2 repeats. The initial OD_{600} cultures were adjusted to have the starting of 0.05.
transformed cells in this study. The explants produced hygromycin resistant tissue within a week of culture on the selection medium and some untransformed dead tissues were also observed (Fig. 4B). The negative control (untransformed) explants cultured on the selection medium did not produce any hygromycin resistant tissues (Fig. 4C). The hygromycin resistant shoot clumps sub-cultured onto the SEM containing 25 mg/l hygromycin and 250 mg/l cefotaxime also produced good response of shoot elongation after 2 weeks of incubation in light (Fig. 4D and E). The hygromycin resistant plants were rooted in the SEM containing 25 mg/l hygromycin and 250 mg/l cefotaxime (Fig. 4F). No *Agrobacterium* overgrowth or contamination during the selection and regeneration was
observed. The plants were recovered and transferred to the greenhouse as mentioned in the plant regeneration results. The plants were then analysed for the confirmation of gene transfer mediated by Agrobacterium.

**Confirmation of transformation by PCR.** The genomic DNA was isolated from hygromycin resistant plants of RNAi lines grown in greenhouse (primary transformants, T0 lines). The presence of the transgene was confirmed using the hygromycin gene specific primers. For each RNAi line, around 35 transgenic plants were obtained from three replicates, each consisting of around 125 explants. The percentage transformation frequency of around 9.0% was obtained for each RNAi line (Table 2). The PCR amplification of 2 representative lines each of these constructs are shown in Fig. 4G. The expected band size of 683 bp is seen in positive control (plasmid; Fig. 4G, lanes 9–11) and for 2 lines each of RNAi transgenic plants (Fig. 4G, lanes 2–7). No band is seen for genomic DNA of non-transformed control plant (Fig. 4G, lane 8), confirming successful integration of the transgene from RNAi vector. These lines were used to develop the T1 progeny which were used for shoot and root morphology analysis, PHT1 transporter expression analysis and Pi uptake studies.

| Name of the phytohormone | Concentration (mg/l) | Percentage of explants responding | Number of shoots/ explant* |
|--------------------------|----------------------|----------------------------------|-----------------------------|
| KN                       | 0.0                  | 0.0                             | 0                           |
|                          | 0.5                  | 36.5                            | 16 ± 1.1 c                  |
|                          | 1.0                  | 28.4                            | 13 ± 0.81 b                 |
|                          | 1.5                  | 19.7                            | 9 ± 0.73 d                  |
|                          | 2.0                  | 13.5                            | 4 ± 0.82 e                  |
|                          | 0.0                  | 0.0                             | 0                           |
|                          | 0.5                  | 95.7                            | 28 ± 0.89 a                 |
|                          | 1.0                  | 81.8                            | 23 ± 0.75 b                 |
|                          | 1.5                  | 38.6                            | 15 ± 1.1 c                  |
|                          | 2.0                  | 32.5                            | 7.0 ± 1.9 d                 |
| BAP                      | 0.0                  | 0.0                             | 0                           |
|                          | 0.5                  | 34.7                            | 18 ± 0.75 b                 |
|                          | 1.0                  | 31.5                            | 14 ± 0.81 c                 |
|                          | 1.5                  | 17.8                            | 6 ± 0.19 d                  |
|                          | 2.0                  | 12.9                            | 3 ± 0.54 c                  |
| TDZ                      | 0.0                  | 0.0                             | 0                           |
|                          | 0.5                  | 34.7                            | 18 ± 0.75 b                 |
|                          | 1.0                  | 31.5                            | 14 ± 0.81 c                 |
|                          | 1.5                  | 17.8                            | 6 ± 0.19 d                  |
|                          | 2.0                  | 12.9                            | 3 ± 0.54 c                  |

Table 1. Effect of phytohormone on multiple shoot induction from shoot apex explants in foxtail millet genotype Maxima. Response was noted after 3 weeks of incubation at 25 ± 2 °C in light. *Values are expressed as mean ± SD of 3 replications and 3 repeats. Values followed by the same letter are not significantly different based on a t-test (P < 0.001).

**SiPHT1;2, SiPHT1;3 and SiPHT1;4 transporters of foxtail millet have distinct, non-redundant roles in Pi transport in planta.** The phenotypic changes and Pi uptake abilities were assessed for T1 lines of RNAi plants grown under low Pi (10 μM). The RNAi lines showed retarded growth when compared to the wild type (control) plants (Fig. 5A and B). Among the 3 transgenic lines, SiPHT1;2-RNAi was much smaller when compared to other two lines (SiPHT1;3-RNAi and SiPHT1;4-RNAi). The photograph shows 2 week old plants grown under low Pi on perlite in the green house (Fig. 5A and B). The length of shoot and primary root and dry weights of shoot and root were determined (Fig. 5C). The RNAi lines showed significantly lower values for length and dry weights of shoot and root when compared to wild type non-transformed plants. SiPHT1;2-RNAi plants showed 50% reduction in shoot length and 26% reduction in root length when compared to the wild type plants. SiPHT1;3-RNAi and SiPHT1;4-RNAi transgenic plants showed only 30% reduction in shoot length and 15% reduction for primary root length when compared to control plants. Similarly significant reduction in dry weights of shoot and root were seen in RNAi plants when compared to wild plants (Fig. 5C), the SiPHT1;2-RNAi lines showed the greatest reduction in both shoot and root dry weights.

The three RNAi lines had lower Pi both in root and shoot tissues confirming the role of these transporters in both uptake of Pi from the soil and transport within the plant. The SiPHT1;2-RNAi lines showed a significantly lower level of Pi when compared to other two lines (P < 0.001). These lines had only one third of the Pi in leaf samples and half of Pi in root samples when compared to control plants that were regenerated without transformation (Fig. 5D). SiPHT1;4-RNAi lines also showed significantly lower Pi in shoot tissues when compared to wild plants but significantly higher than those in SiPHT1;2-RNAi lines. The SiPHT1;3-RNAi lines had shoot Pi that were comparable to that of control plants (no significant difference) but it showed only one third of Pi in root tissues. The Pi content in root tissues of all 3 RNAi lines were also significantly lower when compared to the wild plants (Fig. 5D).

To have tightly controlled supply of external Pi, further experiments were performed in hydroponics. Production of lateral roots and root hairs is an important adaptation to low Pi stress, therefore the lateral root density and root hair density were measured for wild type plants and in each RNAi line grown under low Pi in hydroponics. The images of lateral roots and root hairs of these plants are shown in Fig. 6A and B. The lateral root density (number per 1 cm length) was significantly higher in all RNAi lines when compared to the wild type.
plants under low Pi but the SiPHT1;3-RNAi line produced lateral root density higher than wild type plants but lower than the other 2 RNAi lines (Fig. 6C). Wild type and RNAi lines grown under low Pi produced root hairs with noticeable difference in densities (Fig. 6B). The highest root hair density was observed in SiPHT1;2-RNAi lines (Fig. 6A and B). SiPHT1;3-RNAi and SiPHT1;4-RNAi lines had higher root hair density than wild type plants but lower than the SiPHT1;2-RNAi lines.

Expression pattern of PHT1 genes in T1 seedlings of RNAi lines. The expression pattern of transporters SiPHT1;2, 1;3 and 1;4 were analysed by semi quantitative RT-PCR in all 3 RNAi lines grown on hydroponics under low Pi (10 µM) and compared to that of untransformed (wild type) plants (Fig. 7A). In total, 3 independent transformants were tested for each RNAi line for the reproducibility of results. The root and shoot samples of untransformed (wild type) plants produced the same pattern of expression for these 3 transporters as previously reported. Transporters SiPHT1;2 and 1;4 were found to be expressed in root tissues and transporters SiPHT1;2 and 1;3 were expressed in leaf tissues. However, a little expression of isoform SiPHT1;4 was seen in the leaf sample. The SiPHT1;2-RNAi line showed strongly reduced levels of SiPHT1;2 transcript in root and leaf tissues. No band was seen corresponding to this gene specific RT-PCR product in leaf sample and only a very faint band was seen in root sample (Fig. 7A). Expression of the other 2 genes are not obviously altered in the SiPHT1;2-RNAi line. In the SiPHT1;3-RNAi line, SiPHT1;3 gene specific transcripts were not detected in the root sample and only a very faint band is seen in leaf sample of this transgenic line. The expression patterns of the other genes remain unchanged. This result also confirmed that the RNAi construct designed specifically targeted SiPHT1;3 and downregulated its expression. Bands corresponding to the SiPHT1;4 transcript were not seen in...
Figure 4. *Agrobacterium*-mediated transformation, regeneration using shoot apex explants and confirmation of transformation by PCR in foxtail millet genotype Maxima. Co-cultivation of shoot apex explants on sterile filter papers over MS medium containing 0.5 mg/L BAP and 100 µM acetosyringone after 3 days on incubation in the dark (A), selection of co-cultivated explants on the shoot induction (SIM) medium containing MS salts, 0.5 mg/L BAP, 25 mg/L hygromycin and 250 mg/L cefotaxime, after 2 weeks of incubation in the light (B). The production of hygromycin resistant shoots are visible (B), un-transformed (negative control) explants in the same SIM are dead after 2 weeks of incubation (C), production of hygromycin resistant shoots in the SIM with MS salts, 0.5 mg/L BAP, 25 mg/L hygromycin and 250 mg/L cefotaxime, after 4 weeks of incubation (D), elongation of shoots in the shoot elongation medium (SEM) medium containing MS salts, 25 mg/L hygromycin and 250 mg/L cefotaxime, after one week of incubation in the light (E), rooting of shoots in the same medium after 3 weeks of incubation in the light (F). Confirmation of transformation by detection of *hptII* gene in primary transformants (T0) by PCR (H). Lane 1 and 13 = 1 kb DNA ladder. Lane 2 and 3 = *SiPHT1;2* RNAi lines. Lanes 4 and 5 = *SiPHT1;3* RNAi lines. Lanes 6 and 7 = *SiPHT1;4* RNAi lines. Lane 8 = negative control (genomic DNA of non-transformed plant). Lane 9 = pFGC-*SiPHT1;2* RNAi plasmid (positive control). Lane 10 = pFGC-*SiPHT1;3* RNAi plasmid (positive control). Lane 11 = pFGC-*SiPHT1;4* RNAi plasmid (positive control). Lane 12 = Water control (no template DNA).
either root or leaf tissue of the SiPHT1;4-RNAi line confirming the downregulation of this gene. A faint band was seen in leaf tissues of all other samples but even this band is also not seen in the leaf sample of SiPHT1;4-RNAi line. However downregulation SiPHT1;4 resulted in upregulation of expression of SiPHT1;3 in root samples.

Total and inorganic P contents were reduced in all SiPHT1 RNAi lines. Total and inorganic P contents of leaf and root tissues were analysed in T1 progenies of RNAi lines grown hydroponically under 300 µM (Fig. 7B) and 10 µM Pi (Fig. 7C) respectively. In Fig. 7B and C, the height of the bar represents total P, the lighter shaded area within the bar the proportion of Pi that is inorganic P. The root samples of wild type plants had higher levels of both total and inorganic P than leaves under 300 µM (Fig. 7B). However, RNAi lines showed significantly lower levels of total and inorganic P in both root and leaf tissues under both high and low P conditions (Fig. 7B and C). In the SiPHT1;2-RNAI line grown on 300 µM Pi, both total and inorganic P contents were reduced by around 53% in leaf tissues when compared to wild type plants. In root tissues of this RNA lines, the total P content was reduced by 54% and 76% reduction of Pi compared to wild type plants (Fig. 7B). The SiPHT1;3-RNAI lines showed 24% and 20% reduction in total and inorganic P respectively in leaf samples. However the root samples SiPHT1;3-RNAI lines showed lower levels of both total and inorganic P than leaf samples; 37% reduction in total P and 78% reduction in Pi was seen in root tissues of these RNAI lines. SiPHT1;4-RNAI lines showed moderate reduction (44%) in total P content and with slightly lower levels of Pi (17%) than wild plants in leaf tissues. However, both total and inorganic P contents of root tissues of the same RNAI lines showed much lower levels (67% reduction in total P and 79% reduction in Pi) than wild plants.

The RNAI lines and wild type plants grown in 10 µM Pi showed lower levels for both total and inorganic P contents in root and leaf tissues than those grown under 300 µM Pi. The total P content in leaf and root tissues of wild type plants was almost the same with slightly higher levels of Pi in root than in leaf tissues. All 3 RNAI lines had significantly lower levels of both total and inorganic P content in root and leaf tissues (Fig. 6C). All 3 RNAI lines showed very low levels of total and inorganic P in root tissues compared to leaf tissues. SiPHT1;2-RNAI lines had significantly lower levels of total and inorganic P in leaf tissues than wild plants with 49% reduction when compared to wild plants, the same RNAI lines showed 57% reduction of total Pi and 91% reduction of Pi in root tissues. The total P content in leaf tissues of SiPHT1;3-RNAI and SiPHT1;4-RNAI lines were higher than SiPHT1;2-RNAI lines but lower than wild type plants with significantly different values (P < 0.001). In root tissues, the SiPHT1;3-RNAI and SiPHT1;4-RNAI lines showed 26% and 41% reduction respectively for total P while the Pi contents were reduced to 82% in SiPHT1;3-RNAI line and to 90% reduction in SiPHT1;4-RNAI line (Fig. 6C).

Discussion

Previous work documented the expression profile of the 12 members of the PHT1 phosphate transporter family of foxtail millet. In order to study the in vivo function of this family a novel direct regeneration protocol for foxtail millet using shoot apex explants was developed and combined with Agrobacterium-mediated transformation to generate RNAi lines in which 3 PHT1 transporters SiPHT1;2, SiPHT1;3 and SiPHT1;4 were individually downregulated and the impact on plant growth and phosphate content analysed. Additional evidence for functional activity of SiPHT1;1, SiPHT1;2 SiPHT1;3 SiPHT1;7 and SiPHT1;8 was obtained through heterologous expression and ability to complement a Δpho84 mutant of S. cerevisiae.

On 1 mM Pi, all S. cerevisiae strains grow as Pi can be taken up by low affinity transporters PHO87 and PHO90, the latter being the most important under high Pi conditions. On low (0.2 mM) Pi, growth becomes dependent upon expression of a functional transporter with in vivo function of the PHT1 phosphate transporter family of foxtail millet. In order to study the in vivo function of this family a novel direct regeneration protocol for foxtail millet using shoot apex explants was developed and combined with Agrobacterium-mediated transformation to generate RNAi lines in which 3 PHT1 transporters SiPHT1;2, SiPHT1;3 and SiPHT1;4 were individually downregulated and the impact on plant growth and phosphate content analysed. Additional evidence for functional activity of SiPHT1;1, SiPHT1;2 SiPHT1;3 SiPHT1;7 and SiPHT1;8 was obtained through heterologous expression and ability to complement a Δpho84 mutant of S. cerevisiae. On 1 mM Pi, all S. cerevisiae strains grow as Pi can be taken up by low affinity transporters PHO87 and PHO90, the latter being the most important under high Pi conditions. On low (0.2 mM) Pi, growth becomes dependent upon expression of a functional transporter with in vivo function of the PHT1 phosphate transporter family of foxtail millet. In order to study the in vivo function of this family a novel direct regeneration protocol for foxtail millet using shoot apex explants was developed and combined with Agrobacterium-mediated transformation to generate RNAi lines in which 3 PHT1 transporters SiPHT1;2, SiPHT1;3 and SiPHT1;4 were individually downregulated and the impact on plant growth and phosphate content analysed. Additional evidence for functional activity of SiPHT1;1, SiPHT1;2 SiPHT1;3 SiPHT1;7 and SiPHT1;8 was obtained through heterologous expression and ability to complement a Δpho84 mutant of S. cerevisiae. On 1 mM Pi, all S. cerevisiae strains grow as Pi can be taken up by low affinity transporters PHO87 and PHO90, the latter being the most important under high Pi conditions. On low (0.2 mM) Pi, growth becomes dependent upon expression of a functional transporter with in vivo function of the PHT1 phosphate transporter family of foxtail millet. In order to study the in vivo function of this family a novel direct regeneration protocol for foxtail millet using shoot apex explants was developed and combined with Agrobacterium-mediated transformation to generate RNAi lines in which 3 PHT1 transporters SiPHT1;2, SiPHT1;3 and SiPHT1;4 were individually downregulated and the impact on plant growth and phosphate content analysed. Additional evidence for functional activity of SiPHT1;1, SiPHT1;2 SiPHT1;3 SiPHT1;7 and SiPHT1;8 was obtained through heterologous expression and ability to complement a Δpho84 mutant of S. cerevisiae. On 1 mM Pi, all S. cerevisiae strains grow as Pi can be taken up by low affinity transporters PHO87 and PHO90, the latter being the most important under high Pi conditions. On low (0.2 mM) Pi, growth becomes dependent upon expression of a functional transporter with in vivo function of the PHT1 phosphate transporter family of foxtail millet. In order to study the in vivo function of this family a novel direct regeneration protocol for foxtail millet using shoot apex explants was developed and combined with Agrobacterium-mediated transformation to generate RNAi lines in which 3 PHT1 transporters SiPHT1;2, SiPHT1;3 and SiPHT1;4 were individually downregulated and the impact on plant growth and phosphate content analysed. Additional evidence for functional activity of SiPHT1;1, SiPHT1;2 SiPHT1;3 SiPHT1;7 and SiPHT1;8 was obtained through heterologous expression and ability to complement a Δpho84 mutant of S. cerevisiae.
containing only one phytohormone and minimal subculturing. Direct plant regeneration seems to be an effective approach to minimize somaclonal variation and results in minimal effort for subculturing cycles\(^\text{29}\).

Utilizing the direct regeneration procedure described we also developed a simple and efficient *Agrobacterium*-mediated transformation of foxtail millet. *Agrobacterium*-mediated transformation has been considered as a simple and cost-effective tool for the development of transgenic plants and functional genomics studies. Only a few reports are available for the *Agrobacterium*-mediated transformation of millets\(^\text{22}\). The first study on *Agrobacterium*-mediated transformation of foxtail millet was reported in 2005\(^\text{35}\) using immature inflorescence as explants with the transformation frequency of 6.6%. The same protocol was utilized to study the function of a pollen-specific gene *Sia01* in foxtail millet\(^\text{35}\). Wang et al.\(^\text{34}\) further optimized the regeneration conditions following the *Agrobacterium*-mediated transformation of foxtail millet. However, in all these reports immature inflorescence was used as initial explants and callus mediated regeneration was adopted to recover the transgenic plants. The use of immature inflorescence as initial explants may pose some technical difficulties as these explants are not readily available around the year and needs pre-culture of plants. Further, callus mediated regeneration is a time consuming process and may also induce somaclonal variations. Therefore, the combination of an efficient *Agrobacterium*-mediated transformation of readily available (shoot apex) explants followed by direct regeneration is a significant advance on current methodology. Shoot apex seems to be an excellent source of explant for regeneration and transformation studies in cereals\(^\text{24}\). We have also previously reported an *Agrobacterium*-mediated transformation system for finger millet using shoot apex explants\(^\text{25}\). In this study following such a strategy we have achieved a transformation frequency of >9% which is higher than previous reports. The direct regeneration protocol reported for foxtail millet in this study should lay the foundation for designing similar protocols for other millets and related bio-energy crops like switchgrass. *Agrobacterium*-mediated transformation system has been well established in switchgrass and many important genes were studied in transgenic plants (reviewed in\(^\text{29}\)).

Production of RNAi lines for *SiPHT1*;2, *SiPHT1*;3 and *SiPHT1*;4 allowed investigation of their function in plants. Despite *SiPHT1*;2 having overlapping expression patterns with *SiPHT1*;3 and *SiPHT1*;4, single knock down lines for all 3 genes have clear phenotypes in both root and shoot with respect to growth and tissue Pi concentration, demonstrating that they are not functionally redundant (Figs 5, 6 and 7). This contrasts with results from Arabidopsis where loss of *PHT1*;4 results in a 40% decrease in Pi absorption but only modest changes in internal root Pi\(^\text{27,28}\) and significant phenotypic changes were only seen in double *pht1*;1*Δ pht1*;4 mutants\(^\text{28}\).

In rice *OsPHT1*;8 is expressed in a wide range of tissues and is not induced by low Pi\(^\text{29}\) as is also the case or *SiPHT1*;2 its close neighbour on the phylogenetic tree\(^\text{4}\). RNAi knock down of *OsPHT1*;8 resulted in reduced shoot and root biomass under both high and low phosphate, reduced phosphate uptake and distribution, and reduced phosphate content\(^\text{29}\). Like *SiPHT1*;2, *OsPHT1*;8 could complement a *S. cerevisiae pho84* mutant for growth on low Pi\(^\text{29}\). *OsPHT1*;8 is implicated in redistribution of Pi from source to sink\(^\text{29}\) and it is noteworthy that *SiPHT1*;2 RNAi lines have strongly reduced leaf Pi (Figs 5 and 7). It will be interesting to determine if *SiPHT1*;2 has a role in P allocation to reproductive tissues as *OsPHT1*;8 does\(^\text{29}\).

*SiPHT1*;3 is normally expressed in leaf with a suggestion of upregulation in older leaf on P starvation\(^\text{4}\). *SiPHT1*;3 groups with *OsPHT1*;5 and *OsPHT1*;4 within subfamily IV of the PHT1 transporter\(^\text{8,31}\). *OsPHT1*;5 does not appear to have been characterised in detail. *OsPHT1*;4 is expressed primarily in root and embryo\(^\text{5}3\) but it shows significant upregulation in shoots of older plants on P deprivation and can complement a *S. cerevisiae pho84* mutant\(^\text{13}\) as could *SiPHT1*;3 in this study. Down regulation of *SiPHT1*;3 resulted in reduced root and leaf phosphate under both low and high phosphate compared to wild type and reduced plant stature (Figs 5, 6 and 7). This is in contrast to the *OsPHT1*;4 RNAi mutants which did not show significant changes in height or root length or in root or shoot phosphate under either high or low phosphate regimes\(^\text{33}\).

*SiPHT1*;4 clusters with *OsPHT1*;1 and *OsPHT1*;2 is subfamily IV of the PHT1 transporter\(^\text{8,31}\). *OsPHT1*;1 is constitutively expressed independent of P supply\(^\text{34}\). *SiPHT1*;4 is predominantly expressed in root and shows low Pi dependent up regulation in older leaf therefore these two genes are not orthologous. *OsPHT1*;2 is strongly induced by low P in roots (especially the stele and lateral roots but not epidermal and cortical cells) and only weakly expressed in leaf\(^\text{17}\). Like *SiPHT1*;4 it could not complement a *pho84* mutant but showed low affinity mM range Pi uptake activity when expressed in Xenopus oocyte\(^\text{17}\). Knockdown of *OsPHT1*;2 decreased transport of Pi to the shoot which was reflected in reduced P concentration in shoots\(^\text{17}\), a phenotype also seen in the current study.

Downregulation of *SiPHT1*;4 resulted in the normally leaf expressed *SiPHT1*;3 being upregulated in root samples (Fig. 7A). This effect could be a specific consequence of loss of *SiPHT1*;4 since no change in expression of *SiPHT1*;3 was seen in the *SiPHT1*;2 RNAi plants. This upregulation is also not likely to reflect reduction in intracellular Pi since the reduction is as great or greater in *SiPHT1*;2 RNAi plants but perhaps a perturbation of a systemic signalling pathway. Interestingly several PHT1 transporters in Arabidopsis were shown to be systemically regulated in a split root experiment\(^\text{21}\). Hormones, sugars and microRNAs\(^\text{36}\) are all implicated and interconnected in systemic Pi signalling\(^\text{37}\).

Table 2. Details of transgenic plants obtained and frequency of transformation for each RNAi line. For each treatment, three replicates were maintained. Values are expressed as the mean ± SD.

| Name of the transgenic line | Number of explants used | Total number of transgenic plants obtained (confirmed by PCR) | Frequency of transformation (%) |
|----------------------------|------------------------|-------------------------------------------------------------|--------------------------------|
| *SiPHT1*;2-RNAi            | 126                    | 35                                                          | 9.2 ± 0.58                     |
| *SiPHT1*;3-RNAi            | 125                    | 36                                                          | 9.6 ± 0.37                     |
| *SiPHT1*;4-RNAi            | 121                    | 33                                                          | 9.0 ± 0.49                     |
Root architecture changes have been proposed to be a function of external Pi concentration. However, Pi starved roots show increased sensitivity to auxin due to upregulation of TIR1 promoting lateral root formation. Here, all 3 RNAi lines showed an exaggerated response to low Pi, especially with induction of more lateral roots and root hairs. In contrast, RNAi of *OsPHT1*;1 resulted in no difference in root hair density and a reduction in root hair length in low Pi conditions compared to wild type plants. In Arabidopsis, downregulation of transporters *AtPHT1*;8 and *AtPHT1*;9 produced a similar response to inhibition of primary root length with proliferation of lateral roots and root hairs under low Pi but contrasting results have also been obtained which may reflect complex interactions between phosphate and metal ion homeostasis.

**Figure 5.** Phenotypic variation of foxtail millet seedlings of T1 lines after transformation with *SiPHT1*;2, 3 and 4 RNAi vectors and analysis of Pi content. Three week old seedlings of the 3 RNAi lines and wild type plants grown under low Pi (10 µM) in paper cups containing perlite (A) (bar = 2 cm), seedlings removed from the paper cups showing shoot and root morphology, after 4 weeks of growth (B) (bar = 2 cm), shoot length and primary root length and dry weights of root and shoot of 4 weeks old seedlings of 3 RNAi lines and wild plants grown under low Pi (C), analysis of Pi content in shoot and root tissues of RNAi lines and wild plants grown under low Pi (D). In bar graphs, values are expressed as mean ± SD of 3 replicates (n = 3). Values followed by the same letter are not significantly different (P > 0.001) (separately for shoot length, root length, shoot dry weight, root dry weight and leaf and root Pi).
In summary we established a novel direct regeneration procedure for foxtail millet using readily available shoot apex explants and combined with Agrobacterium-mediated transformation system and characterised RNAi lines for 3 members of the PHT1 gene family. All 3 members showed phenotypes on both high and low Pi demonstrating non-redundancy of function. More detailed characterisation of these RNAi lines and higher resolution study of the expression should shed more light on their roles.

**Figure 6.** Analysis of root morphology in the 3 RNAi lines and wild plants grown under low Pi (10µM) on hydroponics. Whole root images showing the lateral root formation in wild type and 3 RNAi lines grown after 4 weeks (A) (bar = 1 cm), root hair images of wild type plant and 3 RNAi lines after 4 weeks of growth (B), measurement of lateral root density per cm of root and root hair density per 10µM of root in seedlings of wild type plants and 3 RNAi lines grown under low Pi on hydroponics for 4 weeks (C). Values are expressed as mean ± SD of 3 replicates (n = 3). Values followed by the same letter are not significantly different (P > 0.001) (separately for lateral root density shoot and root hair density).
Figure 7. RT-PCR analysis and total and inorganic P content measurement in wild type plants and the 3 RNAi lines. (A) RT-PCR analysis of the 3 RNAi lines grown under low phosphorous (10 µM) using Si-actin, SiPHT1;2, SiPHT1;3 and SiPHT1;4 gene specific primers. WT = wild type plant (untransformed), SiPHT1;2-RNAi = transgenic plant downregulating SiPHT1;2, SiPHT1;3-RNAi = transgenic plant downregulating SiPHT1;3, SiPHT1;4-RNAi = transgenic plant downregulating SiPHT1;4. Lanes 2, 3 and 4 = amplification with SiPHT1;2, SiPHT1;3 and SiPHT1;4 gene specific primers respectively. Except SiPHT1;4-RNAi line, all the gels were cropped from various gels done at different time points. For SiPHT1;4-RNAi line, the individual lanes were from different gels which are indicated by delineating white lines. The individual gels were captured with different exposure times. T1 seedlings (4 weeks old) of RNAi lines grown under high Pi (B) and low Pi (C) were analysed for total and Pi contents. The total and Pi content of 2 weeks old (on hydroponics) seedlings were assayed using the same reported protocol8. The total height of the bars represents the total P. The lighter shading indicates that proportion of the total P that is Pi and the difference is the organic. Values were expressed as mean ± SD of 3 independent RNAi lines grown under low and high Pi. Values followed by the same letter are not significantly different (P > 0.001).
Materials and Methods

Plasmid construction. Plasmid construction for yeast complementation experiment. The coding sequences of PHT1 transporters SiPHT1;1, 1;2, 1;3, 1;4, 1;7 and 1;8 were PCR amplified from genomic DNA (no introns present for these genes) and cloned into pDD-GFP-2 [23] using Spel/AgeI sites to generate C-terminal GFP tagged versions as PHO84-GFP had previously been shown to be functional [43]. The coding sequence of PHO84 of Saccharomyces cerevisiae (ScPHO84) was amplified from the genomic DNA of S. cerevisiae and cloned into pDDGFP-2 using SpeI/Xmal sites. All clones were confirmed by sequencing. The details of cloning primers are given in Supplementary Table S1. All these clones were confirmed by Sanger sequencing (Source Bioscience, UK) before moving onto the yeast complementation experiments. The plasmid maps of these clones were constructed using Vector NTI software (Life Technologies, NY, USA) and are included as Supplementary Figures S5 to S12.

Plasmid construction for RNA interference (RNAi) experiment. Plasmid pFGC1008 obtained from Arabidopsis Biological Resource Centre, USA (ABRC stock number CD3–446) was used to create the S. italicca PHT1-RNAi (SiPHT1-RNAi) plasmids. The plasmid contained two pairs of unique restriction sites (Ascl/Swal and BamHI/Spel) flanking a 335 base pair GUS fragment that serves to separate two components of inverted repeat. Regions of around 200 bp that are unique to each gene were identified by sequence alignment of SiPHT1;2, SiPHT1;3 and SiPHT1;4 and checked by BLAST to avoid the downregulation of any other isoform of SiPHT1 transporters. The 3’ UTR regions were used for downregulating transporters SiPHT1;2 and 4 and an internal coding sequence was used for transporter SiPHT1;3 (Supplementary Figure S13). For cloning, primers were designed (Supplementary Table S2) with both pairs of restriction sites. In the first step of cloning, the amplified target was digested with Ascl/Swal and ligated into the Ascl/Swal-cleaved vector. This plasmid was used as a template for a second cloning with which to complete the inverted repeat construct. The same PCR product used for first digestion was used but this time digested with BamHI/Spel and inserted into the BamHI/Spel sites of the template plasmid and confirmed by sequencing. The plasmid maps of these clones were constructed using Vector NTI software (Life Technologies, NY, USA) and are included as Supplementary Figures (Supplementary Figures S1 to S3). The plasmids were separately mobilized into Agrobacterium strain LBA4404 using freeze thaw method [44] and used for the transformation of foxtail millet.

Yeast complementation assay. For yeast complementation of S. cerevisiae PHO84 mutant (Δpho8; Mata; his3Δ1; leu2Δ0; met15Δ0; ural3Δ0; YML123c::kanMX4) purchased from Euroscarf, Germany (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). All the clones used in the assay were maintained on yeast nitrogen base (YNB) minus Uracil (-URA) plates with raffinose as carbon source. The expression of transporter was induced by the addition of galactose. The assay was performed with YNB without phosphate (Formedium, UK) and -URA broth. Pi was added to the specified concentration from 0.1 M KH2PO4 stock. Acid washed glassware was used throughout. The liquid cultures were initiated with 3 ml YNB-URA broth containing glucose and 1 mM Pi. Single colonies were inoculated and grown overnight at 28°C, 200 rpm. The cells were collected by centrifugation and washed with 5 ml sterile distilled water and finally diluted to 3 ml sterile distilled water. The complementation assay was performed in 30 ml YNB-URA broth cultures containing galactose as carbon source and containing 1 mM or 0.2 mM Pi with the inoculum adjusted to have the starting OD600 of 0.05 for all the cultures. The cultures were grown at 28°C in an orbital shaker with 200 rpm.

Plant tissue culture. The S. italicca genotype ‘Maxima’ (Acc.No: B2 3875) [4] was used for the tissue culture and transformation studies. The seeds were surface sterilized and inoculated in Murashige and Skoog (MS, [45]) basal medium for germination (Hi-Media Mumbai, India). After 3 days of incubation, the shoot apex (4–8 mm in length) were excised inside the laminar flow under aseptic conditions and used for shoot induction. The shoot apices were inoculated on multiple shoot induction medium (SIM) containing MS basal salts supplemented with different concentration of (0.5, 1.0, 1.5 or 2.0 mg/l) BAP, TDZ or KN (Hi-Media, Mumbai, India). The cultures were incubated in light for multiple shoot induction. The shoot clumps containing multiple shoots were transferred onto the shoot elongation medium (SEM) containing MS basal salts alone devoid of any plant growth regulators for elongation of multiple shoots. The shoots were rooted on the SEM after 4 weeks on incubation. The plantlets were separated, roots were washed with sterile water to remove the medium and transferred onto the paper cups containing sterile vermiculite and watered with diluted (10 time) MS basal medium and covered with polythene bags for 2 weeks. The plants were then transferred to the glass house and grown to maturity.

Agrobacterium-mediated transformation of foxtail millet with RNAi plasmids. The shoot apex explants of foxtail millet were used for the transformation with Agrobacterium strain LBA4404 carrying 3 RNAi vectors (pFGC-SiPHT1;2, pFGC-SiPHT1;3 or pFGC-SiPHT1;4). The stepwise protocol for Agrobacterium-mediated transformation is outlined in Fig. 3. On first day 1 (evening), a starter culture of Agrobacterium with plasmid was initiated by inoculation of a single colony of LBA4404 containing the RNAi vector into 3 ml yeast extract peptone (YPE) supplemented with 50 mg/L streptomycin, 35 mg/L chloramphenicol and 10 mg/L rifampicin. The cultures were incubated on a shaker for overnight at 28°C and grown to OD600 = 0.8 to 1.0. On the second day, 100–200 µl of overnight grown starter culture was transferred to 30 ml of YEP (with all 3 antibiotics mentioned above for selection), 100 µM acetylsyringone (AS) was added and the culture grown on the shaking incubator overnight to get an OD600 of 0.6–0.8. On the third day, the bacterial suspension was spun at 10,000 rpm (Eppendorf Centrifuge, Model-5810R, Rotor FA-45–6–30) at 4°C for 10 min and the pellet was re-suspended in liquid SIM (MS + 0.5 mg/L BAP) to obtain a final OD600 = 0.6–0.8. Then 100 µM acetylsyringone (AS) was added. The bacterial suspension was transferred into a sterile 50 ml glass beaker.
Infection of explants. Three days old shoot apex explants were excised and transferred into the 50 ml beaker containing bacterial suspension (40–50 explants/beaker). The beaker was incubated in orbital shaker at 28 °C for 10–15 min at 80 rpm. The explants were then transferred onto a sterile Whatman No.1 filter paper to remove the excess moisture (around 5 min).

Co-cultivation. Infected callus was transferred onto the sterile Whatman No.1 filter paper placed over the co-cultivation medium containing MS + 0.5 mg/L BAP and 100µM AS. The cultures were incubated at 28 ± 2 °C for 3 days in dark. This method of co-cultivation helps to control the overgrowth of Agrobacterium by reducing the moisture and permits better plant recovery on selection.

Selection and regeneration. After 3 days of co-cultivation, the explants were sub-cultured onto the selection medium (SIM containing 25 mg/L hygromycin and 250 mg/L cefotaxime) and incubated at 25 ± 1 °C in light with light intensity of 50 µmol m−2 s−1 photosynthetic photon flux density (PPFD). The cultures were checked regularly for cell death, contamination (if any) and induction of shoots. Any dead explants were removed from the medium to prevent the release of phenolics into the medium. The explants were sub-cultured every 2 weeks into the selection medium. The hygromycin resistant shoots were transferred onto the SEM containing MS basal salts and 25 mg/L hygromycin and 250 mg/L cefotaxime for the recovery of transformed plantlets. The rooted plants were separated, roots were washed with sterile water to remove the medium and transferred onto the paper cups containing sterile vermiculite and watered with 10x diluted MS basal medium, hardened and moved to the greenhouse and grown to maturity as mentioned in the tissue culture section above. The seeds (T1 progeny) from primary transformants (T0) were germinated on MS basal medium containing 25 mg/L hygromycin. The seeds which germinated and established were transplanted in to the green house for further assays.

Confirmation of transformation by PCR. Genomic DNA was isolated from hygromycin resistant plants (primary transformants, T0) and amplified with hptII specific primers (Forward 5’GCCTCATAAGGCAACCAC 3’, Reverse 5’CGAAAAATGTGGACGCGTTC 3’). The PCR was performed in 25 µl reaction mixture containing 100 ng genomic DNA, 2.5 mM MgCl₂, 0.25 mM dNTPs, 200 nM each of forward and reverse primers and 1 U Taq DNA Polymerase (Genet Bio, Daejeon, Korea) in an Eppendorf thermal cycler (Eppendorf Gradient Thermal Cycler, Germany) with an initial denaturation at 95 °C for 5 min followed by 35 cycles of 30 s denaturation at 95 °C, 30 s annealing at 61 °C and 1 min extension at 72 °C with a final extension at 72 °C for 10 min.

Gene expression analysis using RT-PCR. Gene expression in wild type plants and T1 RNAi lines were analysed by RT-PCR based on the method described previously. The details of primers used are listed in Supplementary Table S3. The PCR products were separated on 10% polyacrylamide gels as described previously.

Plant growth experiments and assay of phosphate contents. Inorganic phosphate content was analysed in 4 weeks old seedlings of T1 RNAi lines grown in perlite and supplied with nutrient solution containing 10 µM Pi. The length of shoot and root and dry weights of root and shoot were determined in these seedlings. In order to dictate the more tightly controlled supply of external Pi, T1 plants of RNAi lines were also grown on hydroponics under 10 and 300 µM Pi conditions as mentioned in the previous report, total and inorganic P contents were assayed using the protocol described by previously.

Statistical analysis. The experiments were conducted in a randomized block design. The number of replicates and repeats for each experiment is indicated in the figure legends. Mean values were calculated and the results were analysed on SPSS 16.0 (SPSS Inc., Chicago, IL, USA) using a t-test at the 1% level.

Data availability statement. All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

References
1. Baker, A. et al. Replace, reuse, recycle: improving the sustainable use of phosphorus by plants. Journal of Experimental Botany 66, 3523–3540, https://doi.org/10.1093/jxb/erv210 (2015).
2. Schachtman, D. P., Reid, R. J. & Ayling, S. M. Phosphorus Uptake by Plants: From Soil to Cell. Plant Physiology 116, 447–453, https://doi.org/10.1104/pp.116.2.447 (1998).
3. Cordell, D., Drangert, J.-O. & White, S. The story of phosphorus: Global food security and food for thought. Global Environmental Change 19, 292–305, https://doi.org/10.1016/j.gloenvcha.2008.10.009 (2009).
4. Sattari, S. Z., Bouwman, A. F., Giller, K. E. & van Ittersum, M. K. Residual soil phosphorus as the missing piece in the global phosphorus crisis puzzle. Proceedings of the National Academy of Sciences USA 109, 6348–6353, https://doi.org/10.1073/pnas.1113675109 (2012).
5. Nussaume, L. et al. Phosphate Import in Plants: Focus on the PHT1 Transporters. Frontiers in Plant Science 2, https://doi.org/10.3389/fpls.2011.00083 (2011).
6. Muchhal, U. S., Pardo, J. M. & Raghothama, K. G. Phosphate transporters from the higher plant Arabidopsis thaliana. Proceedings of the National Academy of Sciences USA 93, 10519–10523 (1996).
7. Ceasar, S. A., Baker, A., Muench, S. P., Ignacimuthu, S. & Baldwin, S. A. The conservation of phosphate-binding residues among PHT1 transporters suggests that distinct transport affinities are unlikely to result from differences in the phosphate-binding site. *Biochemical Society Transactions* **44**, 1541–1548, https://doi.org/10.1042/bst20160016 (2016).
8. Ceasar, S. A., Hodge, A., Baker, A. & Baldwin, S. A. Phosphate concentration and arbuscular mycorrhizal colonisation influence the growth, yield and expression of twelve PHT1 family phosphate transporters in foxtail millet (*Setaria italica*). *PLOS ONE* **9**, e108459, https://doi.org/10.1371/journal.pone.0108459 (2014).
9. Bennetzen, J. L. et al. Reference genome sequence of the model plant Setaria. *Nature Biotechnology* **30**, 555–561, https://doi.org/10.1038/nbt.2196 (2012).
10. Zhang, G. et al. Genome sequence of foxtail millet (*Setaria italica*) provides insights into grass evolution and biofuel potential. *Nature Biotechnology* **30**, 549–554, https://doi.org/10.1038/nbt.2195 (2012).
11. Huang, P., Shyu, C., Coelho, C. P., Cao, Y. & Brutnell, T. P. *Setaria viridis* as a model system to advance millet genetics and genomics. *Frontiers in Plant Science* **7**, 1781, https://doi.org/10.3389/fpls.2016.01781 (2016).
12. Ceasar, S. A. & Ignacimuthu, S. Genetic engineering of millets: current status and future prospects. *Biotechnology Letters* **31**, 779–788, https://doi.org/10.1007/s10529-009-9933-4 (2009).
13. Liu, Y. H., Yu, J. J., Zhao, Q. & Ao, G. M. Genetic transformation of millet (*Setaria italica*) by Agrobacterium-mediated. *Agric. Biotechnol. J.* **13**, 32–37 (2005).
14. Wang, M. Z. et al. Culturing of immature inflorescences and Agrobacterium-mediated transformation of foxtail millet (*Setaria italica*). *Afr J Biotechnol* **10**, 16664–16479, https://doi.org/10.5897/Ajb10.2330 (2011).
15. Qin, F. E., Zhao, Q., Ao, G. M. & Yu, J. J. Co-suppression of Si401, a maize pollen specific Zm401 homologous gene, results in aberrant anther development in foxtail millet. *Euphytica* **163**, 103–111, https://doi.org/10.1007/s10681-007-9610-4 (2008).
16. Gillibert, R., Swinnen, E., De Snijder, P., Smets, B. & Winderickx, J. Differential roles for the low-affinity phosphate transporters Pho87 and Pho90 in *Saccharomyces cerevisiae*. *Biochemical Journal* **434**, 243–251, https://doi.org/10.1042/bj20101118 (2011).
17. Ai, P. et al. Two rice phosphate transporters, OsPht1:2 and OsPht1:6, have different functions and kinetic properties in uptake and translocation. *The Plant Journal* **57**, 798–809, https://doi.org/10.1111/j.1365-313X.2008.03726.x (2009).
18. Leggewie, G., Willmitzer, L. & Reisemeier, J. W. Two cDNAs from potato are able to complement a phosphate uptake-deficient yeast mutant: identification of phosphate transporters from higher plants. *The Plant Cell* **9**, 381–392, https://doi.org/10.1105/tpc.9.3.381 (1997).
19. Remy, E. et al. The Phtl:9 and Phlt:8 transporters mediate inorganic phosphate acquisition by the Arabidopsis thaliana root during phosphorus starvation. *New Phytologist* **195**, 356–371, https://doi.org/10.1111/nph.120467.x (2012).
20. Ayadi, A. et al. Reducing the genetic redundancy of Arabidopsis PHOSPHATE TRANSPORTER1 transporters to study phosphate uptake and signaling. *Plant Physiology* **167**, 1511–1526, https://doi.org/10.1104/pp.114.252338 (2015).
21. Shrawat, A. K. & Loré, H. Agrobacterium-mediated transformation of cereal plants: a promising approach crossing barriers. *Plant Biotechnology Journal* **4**, 575–603, https://doi.org/10.1111/j.1467-7652.2006.00209.x (2006).
22. Dosad, S. & Chawla, H. S. In vitro plant regeneration and transformation studies in millets: current status and future prospects. *Indian Journal of Plant Physiology* **21**, 239–254, https://doi.org/10.4103/0971-992X.40502-016-0240-5 (2016).
23. Lakshmanan, P. et al. Developmental and hormonal regulation of direct shoot organogenesis and somatic embryogenesis in sugarcane (*Saccharum spp.* interspecific hybrids) leaf culture. *Plant Cell Reports* **25**, 1007–1015, https://doi.org/10.1007/s00299-006-0154-1 (2006).
24. Sticklen, M. B. & Oraby, H. F. Shoot apical meristem: A sustainable explant for genetic transformation of cereal crops. *In Vitro Cellular & Developmental Biology - Plant* **41**, 187–200, https://doi.org/10.1079/ivp2004616 (2005).
25. Ceasar, S. & Ignacimuthu, S. *Agrobacterium*-mediated transformation of finger millet (*Eleusine coracana* (L.) Gaertn.) using shoot apex explants. *Plant Cell Reports* **30**, 1759–1770, https://doi.org/10.1007/s00299-011-1084-0 (2011).
26. Merrick, P. & Fei, S. Plant regeneration and genetic transformation in switchgrass: A review. *Journal of Integrative Agriculture* **14**, 483–493, https://doi.org/10.1016/S2095-3119(14)60921-7 (2015).
27. Misson, J., Thibaud, M.-C., Bechtold, N., Raghothama, K. & Nussaume, L. Transcriptional regulation and functional properties of Arabidopsis Pht1:4, a high affinity transporter contributing greatly to phosphate uptake in phosphate deprived plants. *Plant Molecular Biology* **55**, 727–741, https://doi.org/10.1007/s11103-004-1965-3 (2004).
28. Shin, H., Shin, H.-S., Dewbre, G. R. & Harrison, M. J. Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low and high-phosphate environments. *The Plant Journal* **39**, 629–642, https://doi.org/10.1111/j.1365-313x.2004.02161.x (2004).
42. Newstead, S., Kim, H., von Heijne, G., Iwata, S. & Drew, D. High-throughput fluorescent-based optimization of eukaryotic membrane protein overexpression and purification in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences USA* **104**, 13936–13941, https://doi.org/10.1073/pnas.0704546104 (2007).

43. Petersson, J., Pattison, J., Kruckenberg, A. L., Berden, J. A. & Persson, B. L. Intracellular localization of an active green fluorescent protein-tagged Pho84 phosphate permease in Saccharomyces cerevisiae. *FEBS Letters* **462**, 37–42, https://doi.org/10.1016/S0014-5793(99)01471-4 (1999).

44. Weigel, D. & Glazebrook, J. Transformation of *Agrobacterium* using the freeze-thaw method. *Cold Spring Harbor Protocols* **2006**, pdb.prot4666, https://doi.org/10.1101/pdb.prot4666 (2006).

45. Murashige, T. & Skoog, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473–497, https://doi.org/10.1111/j.1399-3054.1962.tb08052.x (1962).

46. Doyle, J. J. & Doyle, J. L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**, 11–15, doi:citeulike-article-id:678648 (1987).

47. Chiou, T.-J. et al. Regulation of phosphate homeostasis by MicroRNA in Arabidopsis. *The Plant Cell* **18**, 412–421, https://doi.org/10.1105/tpc.105.038943 (2006).

48. Pound, M. P. et al. RootNav: Navigating images of complex root architectures. *Plant Physiology* **162**, 1802–1814, https://doi.org/10.1104/pp.113.221531 (2013).

49. Slabaugh, E., Held, M. & Brandizzi, F. Control of root hair development in *Arabidopsis thaliana* by an endoplasmic reticulum anchored member of the R2R3-MYB transcription factor family. *The Plant Journal* **67**, 395–405, https://doi.org/10.1111/j.1365-313X.2011.04602.x (2011).

50. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat Meth* **9**, 671–675 (2012).

**Acknowledgements**

This work was supported by the European Union through a Marie Curie International Incoming Fellowship to Dr S. Antony Caesar (Ref. No. FP7-People-2-11-IIIF-Acronym IMPACT-No: 300672 for incoming phase and No. 921672 for return phase). We also thank the late Prof Stephen A. Baldwin, Astbury Centre for Structural Molecular Biology, University of Leeds, UK for all his support and help throughout this study.

**Author Contributions**

S.A.C. conducted experiments. All authors designed experiments and analysed results. S.A.C. and A.B. wrote the paper. All authors read and approved the submitted version.

**Additional Information**

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-14447-0.

**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 indicate if changes were made. This 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