A Novel fry1 Allele Reveals the Existence of a Mutant Phenotype Unrelated to 5'->3' Exoribonuclease (XRN) Activities in Arabidopsis thaliana Roots

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Abstract

**Background:** Mutations in the FRY1/SAL1 Arabidopsis locus are highly pleiotropic, affecting drought tolerance, leaf shape and root growth. FRY1 encodes a nucleotide phosphatase that in vitro has inositol polyphosphate 1-phosphatase and 3',(2'),5'-bisphosphate nucleotide phosphatase activities. It is not clear which activity mediates each of the diverse biological functions of FRY1 in planta.

**Principal Findings:** A fry1 mutant was identified in a genetic screen for Arabidopsis mutants deregulated in the expression of Pi High affinity Transporter 1;4 (PHT1;4). Histological analysis revealed that, in roots, FRY1 expression was restricted to the stele and meristems. The fry1 mutant displayed an altered root architecture phenotype and an increased drought tolerance. All of the phenotypes analyzed were complemented with the AHL gene encoding a protein that converts 3'-polyadenosine 5'-phosphate (PAP) into AMP and Pi. PAP is known to inhibit exoribonucleases (XRN) in vitro. Accordingly, an xrn triple mutant with mutations in all three XRNs shared the fry1 drought tolerance and root architecture phenotypes. Interestingly these two traits were also complemented by grafting, revealing that drought tolerance was primarily conferred by the rosette and that the root architecture can be complemented by long-distance regulation derived from leaves. By contrast, PHT1 expression was not altered in xrn mutants or in grafting experiments. Thus, PHT1 up-regulation probably resulted from a local depletion of Pi in the fry1 stele. This hypothesis is supported by the identification of other genes modulated by Pi deficiency in the stele, which are found induced in a fry1 background.

**Conclusions/Significance:** Our results indicate that the 3',(2'),5'-bisphosphate nucleotide phosphatase activity of FRY1 is involved in long-distance as well as local regulatory activities in roots. The local up-regulation of PHT1 genes transcription in roots likely results from local depletion of Pi and is independent of the XRNs.

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Introduction

In the last ten years, a variety of independent genetic screens have identified defects in the enzyme FIERY1/SAL1 [FRY1]. The first fry1 mutants were identified in a genetic screen based on the deregulation of an ABA reporter gene [1]. FRY1 was described as a repressor of ABA-mediated stress signal transduction, as the corresponding mutant presented an increased sensitivity to cold, salt and drought stresses [1]. FRY1 seems to act as a negative regulator of both ABA-independent and ABA-dependent stress response pathway [2], Estavillo and Pogson, personal communication) and is involved in leaf venation patterning [3]. Independent screens also identified fry1 alleles affecting the regulation of photo-morphogenic processes, including hypocotyl elongation and flowering time [4] and lateral root initiation [5].

Such a diversity of phenotypes could be explained by the complexity of FRY1 activity. FRY1 was originally identified as a bifunctional enzyme presenting both an inositol polyphosphate 1-
phosphatase activity that hydrolyses inositol 1,4,5-trisphosphate (IP3) in vitro, complementing a salt sensitive yeast strain [6], and a highly specific 3',5'-bisphosphate nucleotide phosphatase activity converting PAP (3'-polyadenosine 5'-phosphate) into AMP and phosphate (Pi) [6,7]. This latter activity was predicted to negatively impact the amount of PAP available in the cell. Indeed, in a separate paper that focuses on chloroplast to nuclear signaling in leaves, several authors from the current study shown that PAP content and not inositol phosphates are regulated by Fry1/Sal1 (Estavillo and Pogson, personal communication). In vitro, PAP suppresses the activity of the yeast 5'->3' exoribonucleases Rat1 and Xrn1 [8]. Thus, the accumulation of PAP in loss-of-function cleases Rat1 and Xrn1 [8].

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Using a reporter gene strategy to identify mutations deregulating the expression of the high affinity phosphate transporter PHT1;4 [10], we identified a novel allele of fry1. In addition to root deregulation of the gene reporter, the mutant exhibited strong root architecture defects and a drought resistance phenotype. Through physiological approaches, grafting experiments and mutant analysis, we show that FRY1 plays a role in long distance signaling to roots through its proposed impact on XRN activities in leaves. In contrast, we reveal a new role for FRY1 in the local regulation of phosphate starvation response genes likely linked to a local depletion of Pi in the root stele.

Results and Discussion

Identification of a mutant deregulating PHT1;4::GUS expression and root development

When driven by the promoter of the high affinity phosphate transporter gene PHT1;4, the GUS reporter gene is induced by phosphate starvation and primarily expressed in the Arabidopsis root. We screened seedlings for the deregulation of this root-expressed reporter gene, in an EMS-derived population of a transgenic line. In our screening conditions (i.e. on phosphate-rich medium) the expression of this reporter marker was not detectable in roots of the parental line [10].

Ten day-old seedlings from each of the 1400 M2 families were stained and roots were screened for seedlings with detectable GUS expression [11]. We identified a recessive mutant (fry1-7, see below) that constitutively expressed the GUS reporter gene in the central cylinder and the pericycle of the root and in primary root meristems (Fig. 1A, B). This mutant also displayed shorter primary and lateral roots (Fig. 1C).

Map-based cloning identifies a new allele of fry1

The mutation was mapped on chromosome 5 between microsatellite markers 5.74 and 5.80, which define an approximately 110 kb interval containing 29 genes. A transcriptomic analysis showed that a transcript corresponding to At5g63980 (FRY1/HOS2/SAL1) was down-regulated in the mutant to 30% of the level detected in the PHT1;4::GUS parental line. Sequencing of the corresponding locus in the mutant line revealed a point mutation (G to A exchange) at nucleotide position 559 in the donor site of the second intron of the FRY1 genomic sequence (Fig. 2A). This mutation altered the splicing of FRY1 transcripts, as confirmed by the cloning and sequencing of four FRY1 splice variants in the mutant (Fig. 2B). All splice variants encoded truncated forms of the FRY1 protein, suggesting that this mutant, referred to as fry1-7, is a loss-of-function allele. Expression of the FRY1 cDNA under the control of the 35S promoter in the fry1-7 line complemented the root phenotype (Fig. S1A), confirming that the mutation in fry1 was responsible for the root defect. Importantly, the expression of the GUS reporter was also complemented in PHT1;4::GUS/fry1-7/35S::FRY1 lines (data not shown) and was indistinguishable from the original PHT1;4::GUS line. An allele test between fry1-7 and the T-DNA insertion allele fry1-6 (Fig. 2A; [9]) further confirmed that FRY1 was the causal gene (data not shown).

Together with the root developmental defects, fry1-7 mutants displayed the aerial growth and developmental defects previously described for other fry1 alleles, including fry1-6 [2,4,9]. Young rosette leaves were crinkly and presented rounded leaf margins and shorter petioles (Fig. S1B); whereas older leaves were serrated. In addition, when transferred to soil, the mutant was more tolerant to drought stress than the wild type control (see below) and displayed a general delay in growth (Fig. S1C) and flowering time (data not shown).

fry1 stimulates the transcription of several genes induced by Pi starvation in the stele

To test whether the GUS expression in PHT1;4::GUS/fry1 was due to the upregulation of the endogenous PHT1;4 gene or specific to the T-DNA reporter construct inserted in PHT1;4, we generated a fry1-7 line devoid of any T-DNA insertion by performing a series of back-crosses. We then measured the...
fry1-7

The induction of PHT1;4 was also detected in the fry1-6 allele (Fig. S3), which confirms that the expression of the PHT1;4::GUS transgene in the PHT1;4::GUS/fry1-7 mutant reflects the activation of the endogenous PHT1;4 gene. Thus, in high phosphate conditions, fry1 mutants show a constitutive induction of PHT1;4 in the central cylinder of the root.

We tested whether the fry1 mutation stimulates the expression of other genes related to PHT1;4. This phosphate transporter belongs to a multifaceted family (the PHT1 family) that exhibits a tight co-regulation (in particular during Pi deficiency [12]). We found that PHT1;1 and PHT1;2 (revealed by a common pair of primers), PHT1;7 and PHT1;8 transcripts were also induced in fry1-7 (Fig. 3A, B) as compared to the wild type control.

In order to assay if genes modulated by Pi starvation distinct from PHT1 family could also be affected by fry1 mutation, we tested two other markers associated with Pi deficiency in the stele: Pho1H1 [13] and the At1G73010 phosphatase (Fig. S2). Both genes were found significantly induced in roots of the fry1 background (Fig. 3C and Fig. S4C, D). Analyses revealed an absence of obvious alterations in Pi content, uptake or transport capacity of the fry1 mutant (data not shown). Nevertheless, the levels of gene induction measured here by qRT-PCR are substantially lower than those observed during phosphate starvation [12,14]. This suggested that the reduction of Pi level is probably limited. In addition, such variation should be restricted to the root stele and masked by the accumulation of vacuolar Pi in external root cell layers such as cortex and epidermis. It is therefore not surprising that such specific Pi discrepancies could not be detected by available techniques and only visualized by the use of sensitive reporter genes or by PCR techniques.

Altered root architecture in fry1 mutants is due to reduced meristem activity in the PR and to an LR initiation defect

Alteration of fry1 root architecture has been recently reported [5], but the description of the root phenotype was limited to lateral root initiation. Our analysis indicated that the root system of the fry1-7 mutant is reduced compared to the parental control line both at the primary root (PR) and the lateral root (LR) levels. Seven days post germination (dpg), the fry1-7 mutant primary root was 37% shorter than its parental line, and the fry1-6 primary root was 32% shorter than the Col PR (Fig. 4A). Quantification of PR growth rate during in vitro development in both the fry1-7 and the fry1-6 mutant alleles revealed a statistically significant difference in growth rate when compared with controls (determined by Student’s t test, P<0.01), which likely explains the growth delay observed in the mutant (Fig. 4B).

Reduced root growth can result from a defect in cell elongation and/or from a decrease in meristem activity. Measuring cortical cell length did not reveal any differences between fry1 alleles and wild type controls (Fig. 4C). However, PR cell number in the proximal meristem (PM) at 7dpg [15] was mildly reduced, although statistically significant, in the fry1-6 and fry1-7 mutants when compared to the wild type PM size (Fig. 4D). These results show that the modified PR growth observed in fry1 is due to a defect in maintenance and/or activity of the root apical meristem.

The fry1 mutation also reduced the LR length (Fig. 4E), the LR density (Fig. 4F) and the LR primordia number (Fig. 4G). Thus, it is likely that the altered root architecture of fry1 mutants is not only due to a delay in growth. Interestingly, LR cortical cell length and PM cell number were comparable among fry1-7 and fry1-6 alleles and the corresponding wild type plants when measured at 14 dpg (data not shown), suggesting that an independent factor limits LR initiation or progression. Auxin is a good candidate for such a
factor as the fry1 mutant has reduced auxin sensitivity at the level of LR initiation [5]. Nevertheless, this auxin response defect could not explain all fry1 root traits as the fry1 PR exhibited auxin sensitivity similar to wild type (data not shown).

The 3’,(2’),5’-bisphosphate nucleotide phosphatase activity complements the root mutant phenotype of fry1 as well as the PHT1;4:GUS induction

FRY1 is a bifunctional enzyme whereas AHL (Arabidopsis HAL2-like, At5g54390) is a FRY1 paralog encoding a protein with only the 3’,(2’),5’-bisphosphate nucleotide phosphatase activity in vitro [7]. In order to test whether the 3’,(2’),5’-bisphosphate nucleotide phosphatase activity is sufficient to recover wild type root and PHT1;4 induction level, we used the AHL gene harboring only this activity (i.e. not the inositol polyphosphate 1-phosphatase activity). Overexpression of AHL complemented the root phenotype of the fry1 mutant (Fig. S1D), indicating that the altered root growth of fry1 mutants is likely to be due to the lack of the FRY1 3’,(2’),5’-bisphosphate nucleotide phosphatase activity. In the AHL overexpressor lines, wild type PHT1-4, Pho1H1 and At1g73010 phosphatase transcript levels were re-established (Fig. S3), further confirming the complementation of the fry1 phenotype by AHL activity. As expected, the overexpression of AHL was able to complement the PHT1;4:GUS induction in fry1 (data not shown). These results strongly suggest that the lack of only the 3’,(2’),5’-bisphosphate nucleotide phosphatase activity is responsible for all the phenotypes analyzed in the current study.

Interestingly, Kim and von Arnim [4] showed that the 35S:AHL construct complements the aerial phenotypes of fry1-6. In vivo analysis of PAP and IP3 levels in Col and fry1 mutants (Estavillo and Pogson, personal communication) confirm our conclusion that only the lack of the 3’,(2’),5’-bisphosphate nucleotide phosphatase activity of FRY1, and the concomitant PAP accumulation, are responsible for all fry1 mutant phenotypes described here.

In roots, the FRY1-GFP fusion protein is mainly located in the inner mature tissues and in meristems. The PHT1;4:GUS expression in the internal cell layers of fry1 roots (Fig. 1B) suggests that FRY1 may be expressed in these tissues. To verify this hypothesis, we transformed the PHT1;4:GUS/ fry1-7 mutant line with a GFP-tagged FRY1 genomic construct (pFRY1:FRY1-GFP). This construct is functional because it complemented the root development defects of fry1-7 (data not shown). In the mature part of the roots, the FRY1-GFP fluorescence was detected in all cell layers, except the epidermis (Fig. 5A), with strongly enhanced expression in the pericycle and stele regions of the mature part of the PR. The fusion protein was strongly expressed in the PR meristem and the root cap (Fig. 5B). It was also detected in the LR primordia (Fig. 5C), emerged LR (Fig. 5D) and LR meristems (data not shown). Therefore, the overall FRY1 expression pattern largely overlaps with the PHT1;4:GUS expression pattern observed in a fry1-7 mutant background (Fig. 1B). This suggests that the role of FRY1 on PHT1;4 expression is tissue-specific, as the induction appears limited to the regions where FRY1 shows the highest expression level in planta.
Grafting experiments reveal two modes of action for FRY1

The expression of PHT1;4 and PHT1;4::FRY1 in the root stele led us to examine whether the PHT1;4::GUS induction in fyl1 could be complemented by a mobile component moving from the shoot. We took advantage of the PHT1;4::GUS reporter in our fyl1-7 allele to examine whether FRY1 acts in a tissue-autonomous way. Micrografting experiments were set up with in vitro plantlets (Fig. 6A), using the parental line (PHT1;4::GUS) and the mutant line (PHT1;4::GUS/fry1-7). As expected in high Pi media, we did not observe any GUS expression in roots of the control PHT1;4::GUS/PHT1;4::GUS grafts (Fig. 6B), whereas those of the control PHT1;4::GUS/fry1-7/PHT1;4::GUS/fry1-7 grafts showed strong GUS staining in the central cylinder and the pericycle (Fig. 6C). Grafting a PHT1;4::GUS scion on a PHT1;4::GUS/fry1-7 root stock resulted in roots with the GUS pattern of PHT1;4::GUS plants (Fig. 6E). Therefore, a wild type FRY1 in the shoot does not complement the mutant expression pattern of PHT1;4::GUS in the fyl1-7 root stock.

Then, we tested whether a wild type shoot could complement the root growth phenotype of fyl1 (Fig. 6F-I). Five weeks after grafting, we observed that wild type roots remained small like fyl1 roots (Fig. 6G). Conversely, the fyl1 roots grew like wild type when grafted on a wild type shoot [Fig. 6H]. These grafting experiments indicate that the root growth defect of the fyl1 mutant is complemented by the wild type shoot. We can hypothesize that a mobile component produced only in leaves is necessary in the root pericycle to exhibit normal root growth. When the aerial part of a graft is unable to synthesize this mobile component (fyl1 scion), the roots are less sensitive to auxin and therefore initiate less LR.

To help in the interpretation of these contrasting results, we investigated whether grafting could also restore other known characteristics of fyl1 mutants. Wilson et al. [2] have shown that fyl1 mutants tolerate drought stress up to 50% longer than wild type controls. We used our different graft combinations to test whether this tolerance depends on the root system or on the shoot. Fig. 6J shows that when a wild type scion is grafted on a fyl1 root it is just as tolerant to drought as when it is grafted on a wild type root (p>0.1). In contrast, wild-type root-stocks did not adversely affect the tolerance of fyl1 scions compared to their endogenous roots (p>0.1). By day 12, whatever the grafting combination, most of the wild type scion plants were dead whereas the fyl1 scions survived an additional 3 days on average (p>0.1). These experiments demonstrate that the root genotype does not determine the drought tolerance of the aerial part of the plant, indicating that the lack of FRY1 in the leaves is sufficient for drought tolerance. We therefore investigated whether the drought tolerance phenotype of fyl1 was due to the lack of FRY1 3’(2’),5’-bisphosphate nucleotide phosphatase activity. The fyl1-6/xrn2 xrn3 xrn4 overexpression line displayed a wild type level of drought tolerance (Fig. 7A), indicating that the drought tolerance of fyl1 is due to the lack of 3’(2’),5’-bisphosphate nucleotide phosphatase activity.

The complementation of the fyl1 root development phenotype and drought resistance by a wild type scion and the non-complementation of the PHT1;4::GUS induction by the wild type scion indicates that FRY1 regulates different aspects of plant physiology by two different mechanisms. Presumably, a mobile component produced by leaves expressing FRY1 is moving to roots and regulating root development but not PHT1 expression.

The xrn2 xrn3 xrn4 triple mutant displays the fyl1 lateral root and drought tolerance phenotypes but does not affect primary root

It has been proposed that XRN activity is inhibited in a fyl1 background [9], likely because of the accumulation of the XRNs inhibitor PAP (Estavillo and Pogson, personal communication). Accordingly, both fyl1 and the xrn mutants accumulate RNA intermediates of miRNA-directed post-transcriptional regulation and share common traits [9]. To further analyze the role of XRN in the fyl1 phenotype, we generated an xrn2 xrn3 xrn4 triple mutant that was fertile, unlike the sterile xrn2 xrn3 double mutant. Thus
the triple *xrn2 xrn3 xrn4* mutant facilitated *in vitro* root analysis without antibiotic selection, which has negative consequences on root development. Although the mechanism for the partial phenotypic rescue is unclear, it suggests that *xrn4* mutations act to partially suppress the *xrn2 xrn3* phenotypic effects. We found that the lateral root phenotype of the *xrn2 xrn3 xrn4* triple mutant was similar to that of *fry1* (Fig. 7B), whereas the primary root of the triple mutant was not significantly reduced compared to wild type (Fig. 7C). We also found that the *xrn2 xrn3 xrn4* triple mutant tolerated a drought stress like the *fry1* mutants (Fig. 7A).

Altogether, these results suggest that the pleiotropic phenotype of the *fry1* mutants results, at least in part, from a general perturbation in XRN activities. The *PHT1;4:GUS* induction in *fry1* is unrelated to its action on XRNs

We investigated whether the *xrn* mutations could mimic the induction of *PHT1;4* in *fry1*. For this, we crossed the *PHT1;4:GUS* parental line to the different single, double and triple *xrn* mutant lines. We confirmed the crosses by checking that the GUS marker was active in *L* of the F2 when plants were grown in phosphate deficient media (Table 1). Interestingly, in plantlets grown in complete media, we never observed GUS-stained roots (Table 1) demonstrating that although the *xrn* mutations can mimic many of the *fry1* mutant phenotypes (root architecture, leaf shape, drought tolerance), they do not mimic the induction of the *PHT1;4* locus. In addition, a qRT-PCR analysis of the *xrn2 xrn3 xrn4* triple mutant confirmed that the XRN activities are not responsible for the up-regulation of *PHT1* genes (Fig. S4). Indeed, the assayed mutants (*xrn4-6* and the *xrn2 xrn3 xrn4*) showed the same level of *PHT1;4, PHT1;7, Pho1H1* and *AT1g73019* transcripts as the Col control. Thus, this analysis further confirmed that the *xrn* mutations do not mimic the induction of the *PHT1;4* locus, nor the general induction of phosphate-starvation genes observed in the *fry1* background.

The inability of *xrn* mutants to induce *PHT1;4* transcription argues in favor of a model whereby FRY1 has two physiological roles for the 3',5'-bisphosphate nucleotide phosphatase activity (Modeled in Fig. 8). On one hand, the PAP accumulation in the mutant represses XRN activity, altering various phenotypes linked to the deregulation of the silencing machinery (root architecture, drought tolerance, leaf shape, hypocotyl sensitivity to red light, hormonal sensing and signaling). Indeed, *fry1* late flowering, short petioles and hypocotyl hypersensitivity to red light phenotypes are largely mimicked by the *xrn2 xrn3* double mutant [4]. The root architecture of the *xrn4* single mutant has been described as being similar to that of *fry1* [5]. However, only the *xrn2 xrn3 xrn4* triple mutant presents *fry1*-like lateral root architecture defects in our conditions (Figs. 7B, C). The *xrn4* mutant presents wild type LR development (Fig. S5A) and a PR
length intermediary between the Col and the fry1-6 PR lengths (Fig. S5B). In this mutant, the levels of the phosphate-starvation markers that appear induced in fry1 are comparable to the control levels (Fig. S4). In addition, the xrn4 single mutant is not drought tolerant (Estavillo and Pogson, personal communication). Interestingly, the xrn2 xrn3 drought tolerance level is intermediary between the wild type and the fry1 drought tolerance levels (Estavillo and Pogson, personal communication), whereas the rosette phenotype of the double mutant is similar to that of the fry1-6 mutant [9]. Moreover, both fry1 mutants and the xrn2 xrn3 xrn4 triple mutant tolerate a drought stress that is lethal for the wild type controls (Fig. 7A), even though the rosette shape of the triple mutant is quite different from the fry1 rosette (compare the petiole length in fry1-6 and the xrn2 xrn3 xrn4 triple mutant in Fig. 7A). Thus, the drought tolerance observed in both fry1 and xrn2 xrn3 xrn4 triple mutants is not linked to a reduced leaf biomass.

The F2 progeny of the indicated crosses were grown 7 to 10 days on either a complete or depleted Pi media before the GUS staining. Results on the Pi depleted media serve as a positive control for the presence of the PHT1;4:GUS transgene. Note that on a Pi-rich media, none of the seedlings expressed the GUS reporter gene.

table: Table 1. PHT1;4:GUS expression in different xrn backgrounds.

| Genetic cross          | Number of F2 plantlets stained/total nb of plantlets assayed |
|------------------------|---------------------------------------------------------------|
|                        | on Pi depleted media | on Pi complete media                                      |
| xrn2 X PHT1;4:GUS      | 16/24               | 0/24                                                        |
| xrn3 X PHT1;4:GUS      | 17/24               | 0/24                                                        |
| xrn4 X PHT1;4:GUS      | 34/45               | 0/204                                                       |
| xrn2 xrn4 X PHT1;4:GUS | 39/58               | 0/474                                                       |
| xrn2 xrn3 xrn4 X PHT1;4:GUS | 18/22               | 0/347                                                       |

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and transpiration, but rather to reduced XRN activity. All of the phenotypes linked to perturbations in XRN activities can be complemented by grafting, suggesting the presence of a systemic signal (Fig. 8, left). On the contrary, the induction of phosphate starvation markers is likely linked to a local effect of FRY1 expression (Fig 8, right). It is not complemented by a wild type scion and is not mimicked by the xrn mutations or linked to the root architecture phenotype.

We propose that this effect could be a result of the by-products of FRY1 activity, more specifically the result of the conversion of PAP into AMP + Pi. A reduction of FRY1 activity likely leads to a slight reduction of AMP and phosphate levels (along with an accumulation of PAP) in the tissues where FRY1 is normally very active (the root pericycle, central cylinder and meristems). The reduction of Pi availability would lead to the transcriptional induction of several phosphate starvation genes (including the PHT1;4;GUS marker) in these cell layers. This effect is not complemented by a wild type scion and is not mimicked by the xrn mutations or linked to the root architecture phenotype.

We demonstrate here that FRY1 in shoots controls root development in Arabidopsis. We have identified a novel FRY1 function modulating the transcription of several Pi starvation markers in the root stele. This is the first fry1 mutant phenotype reported to be independent of XRN activities. Instead, it is likely depending on FRY1 impact on the root cytosolic Pi pool, in stele and pericycle cell layers. Interestingly, this phenotype is not complemented by a wild type scion and therefore acts locally.

Materials and Methods

Plant material and growth conditions

The PHT1;4;GUS line (originally referred to as phlt1;4-1 in [10]) was isolated from a T-DNA mutagenized A. thaliana ecotype Wassilewskija (Ws) seeds collection, obtained from INRA [18]. fry1-6 (SALK_020882), xrn2-1 (SALK_041148), xrn3-3 (SAIL_1172C07) and xrn4-6 (SALK_014209) mutants as well as the xrn2 xrn3, xrn2 xrn4 and xrn3 xrn4 double mutants have been described before [9]. Because XRN3 and XRN4 are genetically linked on chromosome 1, whereas XRN2 is on chromosome 5, the xrn2 xrn3 xrn4 triple mutant was generated by crossing xrn2 xrn4 to xrn3 xrn4 so that 1/16 of the F2 plants would be homozygous for the three mutations (xrn2 and xrn3 being genetically independent).

For physiological analyses and RNA extractions, seeds of Col-0, Ws, PHT1;4;GUS, and the different fry1 mutant alleles were cultivated as described before [10]. For drought tolerance tests, plants were grown in individual pots in short days for 4 weeks with standard watering conditions (once a day). Watering was stopped...
for 13 days and the pots were then rehydrated for 3 days before the observations. Alternatively, after the onset of wilting, survival of the plants was quantified by measuring chlorophyll fluorescence as described [2,19].

**Mutagenesis, screening conditions and histology**

Approximately 3000 seeds of *PHT1;4:GUS* were mutagenized with a 0.5% solution of Ethyl methane sulfonate (EMS) as described [20]. Seeds were sown and the M1 plants were cultivated to obtain the M2 generation. Around 30 seeds of each M2 line (1400 lines) were sown in 6-well Petri dishes (NUNC) containing a modified Hoagland medium (1 mM MgSO₄, 2 mM Ca(NO₃)₂, 1.7 mM KNO₃, 1.6 mM MnCl₂, 0.87 mM CuSO₄, 1.7 mM KNO₃, 1.6 mM MnCl₂, 0.87 mM CuSO₄, 0.32 mM ZnSO₄, 0.91 mM K₃PO₄, 1.03 mM Na₂MoO₄, 0.5 mM NH₄H₂PO₄). After 10 days, seedlings were screened for their GUS expression as described [10]. Histological analysis was performed as described [21].

**Genetic analysis and positional cloning of the mutant**

The mutant line was backcrossed three times to the parental line (*PHT1;4:GUS*) to test the linkage of the phenotype to a single Mendelian recessive mutation. For mapping purposes, a mutant plant (*Ws* ecotype) was crossed with a wild-type Col plant. Linkage analysis was performed with the F2 progeny of this cross as described [22]. DNA from F2 seedlings displaying the mutant phenotypes (GUS staining of a root piece from seedlings grown on complete media) was prepared as described [23]. Single Sequence Length Polymorphism markers [24] distributed on the five chromosomes and polymorphic between the *Ws* and *Col* accessions were tested on the extracted DNA. Thermal cycling consisted of an initial denaturation at 94°C for 2 minutes, followed by 30 cycles of denaturation step at 94°C for 15 seconds, annealing at the respective Tm of each oligonucleotide pair for 20 seconds, and extension at 72°C for 45 sec. At the end of the reactions, the PCR products were allowed to extend for 2 minutes at 72°C.

To identify the mutant locus on chromosome 5, the Gramene Simple Sequence Repeat Identification Tool (SSRIT, http://www.gramene.org/db/markers/ssritool) was used to generate new markers in the area surrounding *FRY1*.

**Mutant complementation and tissue localization of *FRY1***

The *FRY1* genomic fragment (1960 bp) and an additional 753 bp upstream region was PCR cloned by standard molecular techniques in the *Ws* accession. After sequencing in the pENTR/D-TOPo (Invitrogen, Carlsbad, USA), an LR clonase (Invitrogen, Carlsbad, USA) reaction was used to clone the genomic fragment in the binary vector pGWB4 [25]. Then, the Arabidopsis *fy1-7* mutant was transformed by a simplified floral dip method [26]. Similar construct were built with the *FRY1* cDNA (1221 bp) with or without a C-terminal GFP fusion, under the control of the 35S promoter. Primary transformants were selected in medium containing 50 μg/L hygromycin. Their progeny was screened for root phenotype and GFP expression in standard in vivo growing conditions using a Leica SP2 AOB5 inverted confocal microscope (Leica Microsystems, Germany) equipped with an Argon ion laser. Prior to confocal observation, plantlets were stained 3 min in 100 μg/mL propidium iodide (PI). Leaf shape, flowering time and GFP expression in mature plants were screened in soil-grown plants, both in short and long days conditions.

**Analysis of root architecture**

Seedlings were photographed at different times after germination and PR and LR length were measured with the ImageJ software (http://rsb.info.nih.gov/ij/). To determine the speed of growth of the main root, photographs were taken at 8, 11 and 14 days post germination (dpg) from which PR length was measured. The daily growth was calculated accordingly. To measure single cell length above the differentiation zone, roots were briefly stained with ruthenium red and observed with a bright field microscope (Leica DMRXA, 20x objective). At least 30 cells for each of 12 different roots per genotype were measured using a micrometric lens. To estimate the size of the proximal meristem (PM), the number of undifferentiated cells in the cortex was measured in at least 30 roots per genotype as described [27,28]. PI-stained roots (3 min in 100 μM PI) were observed by confocal laser scanning microscopy. PI was excited at 514 nm and imaged using a custom 610–720 nm band pass emission filter. To determine the number of LR primordia at different stages of development, we used a Nomarsky optical microscope, as described [29]. All experiments were performed at least three times.

**Grafting experiments**

Grafting was performed according to [30]. Parental and mutant lines were sown in vitro on a MS/10 medium. Four days after sowing they were cut at the hypocotyl level to separate the aerial and root parts. A 0.3 mm diameter silicon ring (Silastic Laboratory tubing, Dow Corning, USA) was used to maintain the aerial seedling scion and the rootstock together to allow fusion. After five days, successful grafts were transferred to fresh medium for 48 hours, followed by GUS staining for 16 h at 37°C. Alternatively, established grafts were put on soil, either on large soil-filled plates or in pots and grown in the greenhouse for 4 weeks in order to assess the root architecture and the drought tolerance of the grafts. For drought tolerance, plants were either cultered in long days (12 h light, 12 h dark), before watering was withheld, then survival of the plants was determined as described [19] or cultered in short days (8 h light, 14 h dark) using a mix of ¼ soil and ¼ sand and an immersion watering per day. Phenotype was assessed after 13 days without watering followed by three days were watering of the individual pots was resumed.

**Molecular and gene expression analysis**

For gene expression analysis, total RNA was extracted from rosettes and roots of 10 day-old plantlets of the *PHT1;4:GUS* parental line and the *PHT1;4:GUS/fy1-7* mutant, grown in MS/10 medium as described previously [12]. cRNA was prepared using the manufacturer’s instructions (www.affymetrix.com support technical manual expression_manual.affx). Labeling and hybridization on the ATH1 microarray and data analysis were performed according to [12]. Microarray data has been deposited at the EMBL database with the accession number E-MEXP-2483 (www.ebi.ac.uk/arrayexpress) and was used in the present work to identify candidate genes during the positional cloning of the mutant locus.

To analyze mRNA splice variants, 10 μg of total RNA from roots and leaves were treated with DNase1 (Roche Diagnostics, Meylan, France) for 15 min at 37°C and were used for the reverse transcription reaction using the AMV Reverse Transcriptase (Roche Diagnostics, Meylan, France) according to the manufacturer’s instructions. Specific primers (sequence available on request) were used to amplify *FRY1* transcripts, both in the wild type and the mutant backgrounds. DNA cloning and sequencing were performed by standard procedures [31].

RTqPCR analyses were performed after reverse transcription (kit from GE Healthcare) and amplification (Applied ABI7000). Primer efficiency factors were measured for each gene and GapC.
and ROC3 were used as reference genes. Primer sequences are available upon request.

**Production of Arabidopsis transformants expressing pAT1G73010::LUC**

A DNA fragment corresponding to 2001 bp of the promoter driving the expression of the AT1G73010 gene (ending right before the ATG) was PCR amplified and cloned into the pEENTRY-D-TOPO vector. The fragment was recombined into the pBGWL7 vector [32] using LR clonase. After sequencing confirmation, the vector was introduced into C58C1 Agrobacterium tumefaciens cells. Arabidopsis plants were transformed using a modified floral dip method [26], and transformed plants were selected using Basta (T1).

Bioluminescence detection was performed on the T2 generation (3-day-old plantlets) using a UPLSAPO 4X dry objective (N.A. 0.16) or a LUCPFLN 40X dry objective (N.A. 0.6) mounted on an Olympus LV200 Luminoview microscope coupled to an ANDOR iKon-M DU934 camera. Images were acquired with an exposure time of 2 min (4X objective) or 4 min (40X objective). Contrast and brightness of the images were adjusted in ImageJ.

**Supporting Information**

**Figure S1** Mutant complementation assays and leaf phenotype of PHT1;4:GUS/fry1-7 mutant. (A) Complementation of the fry1-7 mutant. The progeny of a plant heterozygous for a T-DNA carrying a 35S::FRY1 cDNA construct is shown. Asterisks indicate non-complemented fry1-7 mutant plants that, presumably, did not inherit the transgene. (B) Picture of the rosette of the 3-week-old PHT1;4:GUS line (left) and the PHT1;4:GUS/ fry1-7 mutant (right) grown on soil under short day conditions. (C) fry1-6/fry1-7 mutant (middle) and the complemented line (A) Quantitative real time PCR of the PHT1;4 transcripts in Col, fry1-6 and fry1-6/35S::AHIL roots. (B) Quantitative real time PCR on the Pho1H1 transcript in Col, fry1-6 and fry1-6/35S::AHIL roots. (C) Quantitative real time PCR on the At1g73010 phosphatase transcript in Col, fry1-6 and fry1-6/35S::AHIL roots. Biological triplicates were performed and all samples were analyzed with technical triplicates. White bars correspond to Col roots, grey bars to fry1-6 roots and black bars to fry1-6/35S::AHIL roots. Standard deviations are shown.

**Figure S2** The pAT1G73010::LUC construct reveals the stelar specificity of gene expression in Arabidopsis roots and the phosphate starvation induction. (A) Transmitted light image and (B) bioluminescence signal of pAT1G73010::LUC plantlets grown for 4 days on P depleted medium then for 4 days on complete medium (plantlet on the left) or for 8 days on P depleted medium (plantlet on the right). Scale bar, 1 mm. (C) Close up of a mature part of a root from a plantlet grown for 8 days on P depleted medium (overlay of transmitted light and bioluminescence signal). The bioluminescence signal is only detected in the central cylinder. Scale bar, 100 μM.

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